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B.Sc. in Biotechnology and M.Sc. in Molecular Biomedicine

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A mis padres

The IFI16 protein (gamma interferon-inducible protein 16) is a DNA sensor that recognizes pathogenic DNA both in the cytoplasm and the nucleus. Activation of IFI16 after infection with a range of viruses induces the production of type I IFNs and the formation of an inflammasome complex that leads to the maduration of IL-1β. In addition to its role as an innate immune receptor, IFI16 also participates in the DNA damage response due to its physical interaction with the tumour suppressor proteins p53 and BRCA1. In particular, IFI16 collaborates with BRCA1 in the induction of p53-mediated apoptosis in response to DNA damage. The initial aim of this thesis was to study whether the functions of IFI16 in these contexts of cancer and viral infection were related.

Initial experiments showed that the levels of IFI16 protein were greatly diminished during herpes simplex virus type 1 (HSV-1) infection. During the course of this thesis other groups published describing this phenomenon, however, the mechanisms underlying the degradation of IFI16 and, in particular, the role of the HSV-1 ICP0 protein in this process remained controversial. Thus here, the effect of ICP0 on IFI16 expression levels was reassessed and this work demonstrated that ICP0 is required to efficiently trigger IFI16 loss, although the degree of reduction of IFI16 expression varied between different cell lines.

These observations posed the question of how HSV-1 infection might affect other proteins known to interact with IFI16 and the HSV-1 genome, such as p53 and BRCA1. p53 expression was not consistently affected by HSV-1 infection in the cell lines analyzed, confirming previous publications. Surprisingly, however, analysis of BRCA1 expression during HSV-1 infection revealed a marked increase in a protein of approximately 120 kDa. This phenotype was observed in all the cell lines examined and was specific for HSV-1 infection. Since full-length BRCA1 has an apparent molecular weight of around 220 kDa, a proteomic characterization of this 120 kDa was undertaken to definitively establish its identity. These experiments revealed that the 120 kDa protein up-regulated during HSV-1 infection was the viral tegument protein UL37 and further experiments rigorously confirmed this result. A number of BRCA1-reactive antibodies show cross-reactivities with other cellular proteins, but this is the first description of the cross-reactive binding of a BRCA1 antibody to a viral protein. These data emphasise the importance of careful characterisation of antibody reagents for correct interpretation of immunochemical data.

Since IFI16 enhances p53 transcriptional activity we wondered whether p53 in turn might regulate IFI16 expression or activity. The observation that IFI16 expression was markedly reduced in a p53 deficient subline of the HCT116 colorectal carcinoma cell line was consistent with this hypothesis. However, restoration of p53 expression had no appreciable effect on IFI16 expression. Similarly, analysis of a panel of breast cancer cell lines revealed that although the loss of IFI16 expression was common, no clear relation between IFI16 expression and p53 status was found.

La proteína IFI16 (del inglés *gamma interferon-inducible protein 16*) es un sensor de ADN que reconoce ADN patogénico tanto en el citoplasma como en el núcleo celular. La activación de IFI16 tras la infección por un amplio espectro de virus induce la producción de IFNs de tipo I y la formación de un complejo proteico, llamado inflamasoma, que desencadena la maduración de IL-1β. Además de su papel como receptor del sistema inmunitario innato, IFI16 también participa en la respuesta al daño en el ADN debido a su capacidad de interaccionar directamente con las proteínas supresoras de tumores p53 y BRCA1. En particular, IFI16 colabora con BRCA1 en la inducción de la apoptosis mediada por p53 en respuesta a daño en el ADN. El objetivo inicial de esta tesis consistió en estudiar si las funciones de IFI16 se encontraban relacionadas en los contextos de cáncer e infección viral.

Experimentos iniciales mostraron que los niveles de la proteína IFI16 se reducían marcadamente durante la infección por el virus herpes simple de tipo 1 (en inglés HSV-1). Durante el transcurso de esta tesis otros grupos describieron este fenómeno, sin embargo, el mecanismo que subyace a esta degradación y, en particular, el papel de la proteína ICP0 del HSV-1 en este proceso, ha sido objeto de controversia. Por lo tanto, en este trabajo se decidió revisar el efecto de ICP0 en la expresión de IFI16, y demostramos que ICP0 es necesaria para desencadenar una reducción eficiente en los niveles de IFI16, aunque el grado de esta disminución varía entre líneas celulares.

Esta observación nos llevó a plantearnos cómo podría el HSV-1 estar afectando a otras proteínas que interaccionan con IFI16 y el genoma del virus, como p53 y BRCA1. En las líneas celulares analizadas la expresión de p53 no se vio afectada de manera consistente, lo que confirma publicaciones previas. Sorprendentemente, sin embargo, el análisis de la expresión de BRCA1 durante la infección por el HSV-1 mostró un importante aumento de una proteína de aproximadamente 120 kDa. Este fenotipo se observó en todas las líneas celulares analizadas y fue específico de la infección por el HSV-1. Puesto que la proteína BRCA1 canónica tiene un peso molecular aparente de 220 kDa, se llevó a cabo una caracterización por proteómica de la banda de 120 kDa para determinar su identidad. Estos experimentos revelaron que la proteína de 120 kDa que aumenta sus niveles tras la infección por HSV-1 era la proteína del tegumento viral UL37, como confirmaron rigurosamente experimentos adicionales. Se sabe que una serie de anticuerpos frente a BRCA1 muestran reactividad cruzada con otras proteínas celulares, pero nuestros datos representan la primera descripción de una reactividad cruzada entre un anticuerpo contra BRCA1 y una proteína viral. Estos datos subrayan la importancia de realizar una cuidadosa caracterización de los anticuerpos para una correcta interpretación de los datos de inmunoquímica.

Dado que IFI16 potencia la actividad transcripcional de p53, nos planteamos si éste, a su vez, podría regular la expresión o actividad de IFI16. El hecho de que la expresión de IFI16 se viera muy reducida en la línea celular de carcinoma colorrectal HCT116 deficiente en p53 era consistente con esta hipótesis; sin embargo la restauración de la expresión de p53 no tuvo efecto apreciable en la expresión de IFI16. De manera similar, el análisis de un panel de líneas celulares de cáncer de mama mostró que aunque la pérdida de la expresión de IFI16 es común, no se aprecia una relación clara entre la expresión de IFI16 y el estado de p53.

Abbreviations

Abbreviation	Description
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Vero	“verda reno”,”green kidney” in speranto. African green monkey cells.
p21	21 kilodalton cellular protein fraction
TKM buffer	25 mM Tris-HCl pH 7.4, 5 mM KCl, 1 mM MgCl2
p53	53 kilodalton cellular protein fraction
AIM2	Absent in melanoma 2
AP-1	activator protein-1
AR	Androgen Receptor
ASC	apoptosis-associated speck-like protein containing a CARD domain
ATM	ataxia-telangiectasia mutated
ATRX	ATP-dependent helicase alpha thalassemia/mental retardation syndrome X-linked
BHK	baby hamster kidney
BLAST	Basic Local Alignment Search Tool
Bcl-2	B-cell lymphoma 2
Bax	Bcl-2-associated X protein
BRCT2	BRCA1 C Terminus domain 2
BASC	BRCA1-associated genome surveillance complex
BARD1	BRCA1-associated RING domain protein 1
BRCA1	breast cancer type 1 susceptibility protein
BT-474	breast tumor cell line 474
BT-549	breast tumor cell line 549
CARDIF	CARD adapter inducing interferon beta
COSMIC	Catalogue of Somatic Mutations in Cancer
CADM3	cell adhesion molecule 3
CA	cell-associated
cGAS	cGAMP synthethase
chk2	Checkpoint kinase 2
CRM1	chromosome region maintenance 1
CD34	Cluster of Differentiation 34
CLRs	C-type lectin receptors
Cos-7	CV-1 (simian) in Origin, carrying SV40
cGAMP	cyclic di-GMP-AMP
c-di-AMP	cyclic-diadenosine monophosphate
c-di-GMP	cyclic-diguanosine monophophate
DAMPs	damage-associated molecular patterns

DHX36	DEAH (Asp-Glu-Ala-His) Box Helicase 36
DHX9	DEAH (Asp-Glu-Ala-His) Box Helicase 9
DDX	DExD/H-box helicase
DDX41	DExD/H-box helicase 41
DTT	dithiotreitol
DDR	DNA damage response
DAI	DNA-dependent activator of IRFs
DNA-PK	DNA-dependent protein kinase
Mdm2	Double minute 2 protein
dsDNA	double-strand DNA
DMEM	Dulbecco's modified Eagle's medium
E2F	E2 transcriptional factor
E genes	early genes
EGFR	epidermal growth factor receptor
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
FADD	FAS-associated death domain protein
c-Fos	FBJ murine osteosarcoma viral oncogene homolog
FcR	Fc-receptor
FBS	fetal bovine serum
FACS	fluorescence activated cell sorting
LacZ	Gene Z of lac operon
G418	Geneticin
GR	glucocorticoid receptor
gI	Glycoprotein I
Gy	Gray
GFP	Green Fluorescent Protein
HeLa	Henrietta Lacks
HSV-1	herpes simplex virus type I
HSV-2	herpes simplex virus type II
HVEM	herpesvirus entry mediator
HMGBs	High-mobility group box proteins
HR	homologous recombination
HRP	horseradish peroxidase
HCF-1	host cell factor-1
hpi	hours post infection
HCMV	human cytomegalovirus
hDaxx	human death domain-associated protein 6

HEK293T	human embryonic kidney clone 293 SV40 large T antigen
HEL	human embryonic lung cells
HER1	human epidermal growth factor receptor 1
HER2	human epidermal growth factor receptor 2
HER4	human epidermal growth factor receptor 4
HFs	human fibroblasts
HFF	human foreskin fibroblasts
HepaRG	human hepatocellular carcinoma cell line
HHV	human herpesviruses
HIV	human immunodeficiency virus
U2OS	human osteosarcoma
HPV	human papilloma virus
hTERT	human telomerase reverse transcriptase
ΔICP0	ICP0-null virus
ISG15	IFN-stimulated gene 15
ISGs	IFN-stimulated genes
IE genes	immediate early genes
IF	Immunofluorescence
IgG	immunoglobulin G
IP	Immunoprecipitation
ICP0	Infected Cell Polypeptide 0
ICP22	Infected Cell Polypeptide 22
ICP27	Infected Cell Polypeptide 27
ICP4	Infected Cell Polypeptide 4
ICP47	Infected Cell Polypeptide 47
IFN	interferon
IPS-1	Interferon beta promoter stimulator protein 1
IFI200 family	interferon inducible 200 family
IRF3	interferon regulatory factor 3
IRF5	interferon regulatory factor 5
IRF7	interferon regulatory factor 7
IRFs	Interferon regulatory factors
IFI16	gamma interferon-inducible protein 16
ISREs	Interferon-Stimulated Response Elements
IL-18	Interleukin 18
IL-1β	Interleukin 1β
IARC	International Agency for Research on Cancer
ICTV	International Committee on the Taxonomy of Viruses

KSHV	Kaposi's sarcoma-associated herpesvirus
kDa	kilo Dalton
L genes	late genes
LRRFIP1	Leucine-rich repeat flightless-interacting protein 1
LSm14A	Like-Sm protein 14A
UL30	long unique region 30
UL37	long unique region 37
UL42	long unique region 42
UL47	long unique region 47
MHC	major histocompatibility complexmajor histocompatibility complex
MS	mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
Mdc1	Mediator of DNA damage checkpoint protein 1
MP	Medium Purified
MRE11	meiotic recombination 11 protein
SK-OV3	Memorial Sloan Kethering Ovarian Carcinoma 3
SK-BR-3	Memorial Sloan–Kettering Cancer Center Breast 3
MCF10A	Michigan Cancer Foundation-10
MCF7	Michigan Cancer Foundation-7
MAVS	Mitochondrial antiviral-signaling protein
MRN	Mre11-Rad50-Nbs1 complex
m.o.i.	multiplicity of infection
MNDA	Myeloid cell nuclear differentiation antigen
MyD88	myeloid differentiation primary response 88
NALP3	NACHT, LRR and PYD domains-containing protein 3
nm	nanometers
Nbs1	Nijmegen breakage syndrome protein 1
NLRP3	NOD-like receptor family, pyrin domain containing 3
NHEJ	non-homologous end-joining
NP-40	Nonidet P-40
ND10	nuclear domains 10
NES	nuclear export signals
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	nuclear localization signal
NLRs	nucleotide-binding oligomerization domain (NOD)-like receptors
Oct-1	Octamer-binding protein 1
OB	oligonucleotide/oligosaccharide binding
p53RE	p53 responsive elements

53BP1	p53-binding protein
pfu	particle forming units
PAMPs	pathogen-associated molecular patterns
PRRs	pattern recognition receptors
PAXIP1	PAX-interacting protein 1
PCM1	Pericentriolar material 1
PMSF	phenylmethylsulfonyl fluoride
PBS	phosphate-buffered saline
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PVDF	polyvinylidene fluoride
PML	promyelocytic leukemia protein
PYCARD	PYD and CARD domain-containing protein
PYHIN	pyrin and HIN domain-containing
PYHIN1	Pyrin and HIN domain-containing protein 1
PYD	pyrin domain
POP3	Pyrin domain only protein 3
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
RING	Really Interesting New Gene
pRb	retinoblastoma protein
RLRs	retinoic acid inducible gene-I (RIG-I)-like receptors
RIG-I	retinoic acid-inducible gene 1
rpm	revolutions per minute
RNF168	RING finger protein 168
RNF8	RING finger protein 8
RT	Room temperature
RPMI	Roswell Park Memorial Institute
Saos2	Sarcoma osteogenic
Ser15	Serine 15
(S-T-P)-rich spacer	serine-threonine-proline -rich spacer
ssDNA	single stranded DNA
SUMO	small ubiquitin-like modifier
SDS	sodium dodecyl sulfate
NaF	Sodium Fluoride
Na3VO4	Sodium Orthovanadate
Sp100	speckled protein 100kDa
STING	stimulator of interferon genes protein
TBK1	TANK-binding kinase 1

TLR9	Toll-like receptor 9
TLRs	Toll-like receptors
TMEM173	Transmembrane Protein 173
TAP	transporter associated with antigen processing
TAE buffer	Tris base, acetic acid and EDTA.
UV	Ultraviolet
VACV	vaccinia virus
VSZ	Varicella Zoster Virus
gE	viral glycoprotein E
vhs	virion host shutoff protein
VISA	Virus-induced-signaling adapter
wt	wild type
ZBP1	Z-binding protein 1
α-TIF	α gene trans-inducing factor
gD	Glycoprotein D
gH	Glycoprotein H
IFIX	interferon inducible protein X
MEK1/2	MAPK/ERK kinase 1/2
TBS	tris buffer solution

Introduction

1. DNA sensors

The key signals that initiate the **innate immune responses** are mediated by cellular germline-encoded pattern recognition receptors (**PRRs**) that detect microbial structurally conserved motifs known as pathogen-associated molecular patterns (**PAMPs**) or endogenous ligands released under stress called damage-associated molecular patterns (**DAMPs**). Several families of PRRs, such as Toll-like receptors (**TLRs**) (reviewed in (O'Neill, Golenbock et al. 2013)), nucleotide-binding oligomerization domain (NOD)-like receptors (**NLRs**) (reviewed in (Barbe, Douglas et al. 2014)), C-type lectin receptors (**CLRs**) (reviewed in (Hoving, Wilson et al. 2014)), retinoic acid inducible gene-I (RIG-I)-like receptors (**RLRs**) (reviewed in (Loo and Gale 2011)) and certain **DNA sensors** (reviewed in (Paludan and Bowie 2013)), have already been identified. Stimulation of these receptors triggers signalling pathways, involving the activation of transcription factors such as NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) and IRFs (Interferon regulatory factors), which culminate in the induction of expression of host inflammatory cytokines, chemokines or interferons (IFNs). These effector molecules also contribute to the initiation and development of pathogen-specific adaptive immune responses (reviewed in (Medzhitov 2007)).

IFNs are a family of cytokines known to play a crucial role in **host defense against viral infections** and in additional activities including immunomodulation and regulation of cell growth and differentiation in a variety of cell types (reviewed in (Gutterman 1994; Goodbourn, Didcock et al. 2000)). The three classes of IFNs identified, type I (α and β), type II (γ) and type III (λ), regulate cellular activities through the binding to their specific cell surface receptors that activate the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway leading to the expression of a wave of hundreds of IFN-stimulated genes (ISGs), whose products mediate distinct biological functions including antiviral activities (reviewed in (Sadler and Williams 2008)). Specifically, type I IFNs are known to be essential for a robust antiviral response (reviewed in (Ivashkiv and Donlin 2014)).

Although the PAMPs that have been best described are microbial proteins, lipids, carbohydrates and RNA, it is well-known that DNA is also a potent immunostimulatory molecule. Detection of **pathogen-derived DNA** is one major mechanism of **type I IFN induction** (Tokunaga, Yano et al. 1992; Ishii, Coban et al. 2006; Stetson and Medzhitov 2006), and generally involves signalling via the stimulator of interferon genes protein (**STING**), which triggers TANK-binding kinase 1 (**TBK1**)-mediated activation of interferon regulatory factor 3 (**IRF3**). Thus, STING (also called MITA, ERIS, TMEM173) functions as a central adaptor protein between detection of DNA and the TBK1/IRF3 signaling axis (Ishikawa and Barber 2008; Zhong, Yang et al. 2008; Ishikawa, Ma et al. 2009; Tanaka and Chen 2012).

The PRRs known to be able to sense the presence of pathogen-derived intracellular DNA include several proteins with differential cell population distribution and subcellular localization. These DNA sensors are located within the cell at vesicles in the cytoplasm, within the cytoplasm or within the

nucleus (reviewed in (Nie and Wang 2013; Paludan and Bowie 2013; Orzalli and Knipe 2014)) (Figure I1).

The first DNA sensor identified was **TLR9** (Hemmi, Takeuchi et al. 2000), a member of the TLR family that is expressed in the endoplasmic reticulum and translocated to the membrane of endosomal and lysosomal compartments for ligand binding and signal transduction (Latz, Schoenemeyer et al. 2004). TLR9 leads to type I IFN induction via the adaptor protein MyD88 (myeloid differentiation primary response 88) and IRF7 (Kawai, Sato et al. 2004).

The DNA-dependent activator of IRFs (**DAI**) protein (also called ZBP1, Z-binding protein 1) was the first cytosolic DNA sensor identified, which induces the production of type I IFN in a TBK1-IRF3-dependent pathway (Takaoka, Wang et al. 2007).

The second cytosolic DNA sensor identified was **RNA polymerase (pol) III**, which transcribes AT-rich dsDNA to produce 5'-triphosphate dsRNA that is recognized by **RIG-I**, a DExD/H-box helicase (DDX) protein that belongs to the RLR family of PRRs, which subsequently initiates the production of type I IFN (Ablasser, Bauernfeind et al. 2009; Chiu, Macmillan et al. 2009) via a pathway that includes the adaptor protein MAVS (Mitochondrial antiviral-signaling protein), also called IPS-1 (Interferon beta promoter stimulator protein 1), VISA (Virus-induced-signaling adapter) or CARDIF (CARD adapter inducing interferon beta), which subsequently triggers activation of the TBK1-IRF3 pathway leading to type I IFN genes transcription (Kawai, Takahashi et al. 2005; Meylan, Curran et al. 2005; Seth, Sun et al. 2005; Xu, Wang et al. 2005).

Other members of the DDX protein family have also been implicated in cytosolic DNA sensing, such as **DHX9** (DEAH box protein 9) and **DHX36** (DEAH box protein 36), which interact with the MyD88 adaptor protein (Kim, Pazhoor et al. 2010), as well as **DDX41** (DEAD box protein 41) (Zhang, Yuan et al. 2011b) that depends on STING to induce type I IFN. DDX41 has also been involved in the binding of the bacterial secondary messengers cyclic-diguanosine monophophate (c-di-GMP) and cyclic-diadenosine monophosphate (c-di-AMP). Upon recognition of these bacterial dinucleotides, DDX41 interacts with STING to induce the expression of type I IFNs (Parvatiyar, Zhang et al. 2012). Additionally, c-di-GMP and probably c-di-AMP also bind to STING (Burdette, Monroe et al. 2011; Barker, Koestler et al. 2013).

cGAS (cGAMP synthethase) has recently been identified as a cytosolic DNA sensor that in response to DNA catalyzes the synthesis of cyclic di-GMP-AMP (cGAMP). The endogenous cGAMP, whose structure resembles the bacterial cyclic dinucleotides, directly binds to and activates STING (Sun, Wu et al. 2013; Wu, Sun et al. 2013).

STING, in addition to its capacity to bind cyclic dinucleotides and its established role as a key adaptor in the IFN response for most DNA sensors, has also been reported to function as a direct sensor of DNA (Abe, Harashima et al. 2013).

Additionally, some key components of the DNA damage repair machinery are involved in the detection of immunostimulatory DNA. For example, DNA-dependent protein kinase (**DNA-PK**), a heterotrimeric protein complex consisting of Ku70, Ku80 and the catalytic subunit DNA-PKcs

(reviewed in (Smith and Jackson 1999)), and the meiotic recombination 11 protein (**MRE11**) have also been identified as cytosolic DNA sensors (Zhang, Brann et al. 2011a; Ferguson, Mansur et al. 2012; Kondo, Kobayashi et al. 2013). Both proteins induce immune responses in a STING-dependent manner (Ferguson, Mansur et al. 2012; Kondo, Kobayashi et al. 2013).

Other proteins that have been proposed to function as cytosolic DNA sensors are **LRRFIP1** (Leucine-rich repeat flightless-interacting protein 1) (Yang, An et al. 2010)**, HMGBs** (High-mobility group box proteins) (Yanai, Ban et al. 2009) and **LSm14A** (Like-Sm protein 14A) (Li, Chen et al. 2012b).

In addition to the activation of the STING-dependent signalling pathway, which results in the induction of type I IFNs, innate recognition of pathogenic DNA also leads to the assembly of caspase-1-activating multiprotein complexes termed **inflammasomes** that promote the proteolytic processing of certain proinflammatory cytokines into their mature active and secreted forms (Muruve, Petrilli et al. 2008).

An inflammasome is composed of a **PRR**, the adaptor molecule **ASC** (apoptosis-associated speck-like protein containing a CARD domain) and the **procaspase-1**. The adaptor ASC, also known as PYCARD (PYD and CARD domain-containing protein), provides a link between the specific PRR and the procaspase-1 through homotypic interactions via the PYD and CARD domains. Upon PRR activation, its PYD domain interacts with the PYD domain of ASC, whose CARD domain recruits the CARD of procaspase-1 permitting the autocleavage and formation of the active caspase-1 p10/p20 tetramer, which in turn cleaves proinflammatory cytokines, such as interleukins IL-1β and IL-18, leading to their maturation (reviewed in (Rathinam, Vanaja et al. 2012)) (Figure I1).

PRRs able to sense DNA and activate inflammasome signalling include **NLRP3** protein (NOD-like receptor family, pyrin domain containing 3), also called NALP3 (NACHT, LRR and PYD domains-containing protein 3), which is a member of the NLR family of PRRs. It senses a large range of molecules including cytosolic microbial DNA and subsequently forms an ASC-dependent inflammasome resulting in the activation of caspase-1 and hence the maturation of IL-1β (Muruve, Petrilli et al. 2008). **AIM2** (Absent in melanoma 2), a member of the PYHIN (pyrin and HIN domain-containing) protein family, recognizes cytoplasmic DNA and forms an inflammasome complex that leads to the release of IL-1β (Burckstummer, Baumann et al. 2009; Fernandes-Alnemri, Yu et al. 2009; Hornung, Ablasser et al. 2009). Another member of the PYHIN family of proteins, the gamma interferon-inducible protein 16 (**IFI16**) has also been described to function as a DNA sensor, acting both in the cytosol and the nucleus, which is involved in STING-mediated type I IFN production (Unterholzner, Keating et al. 2010) as well as inflammasome signalling (Kerur, Veettil et al. 2011).

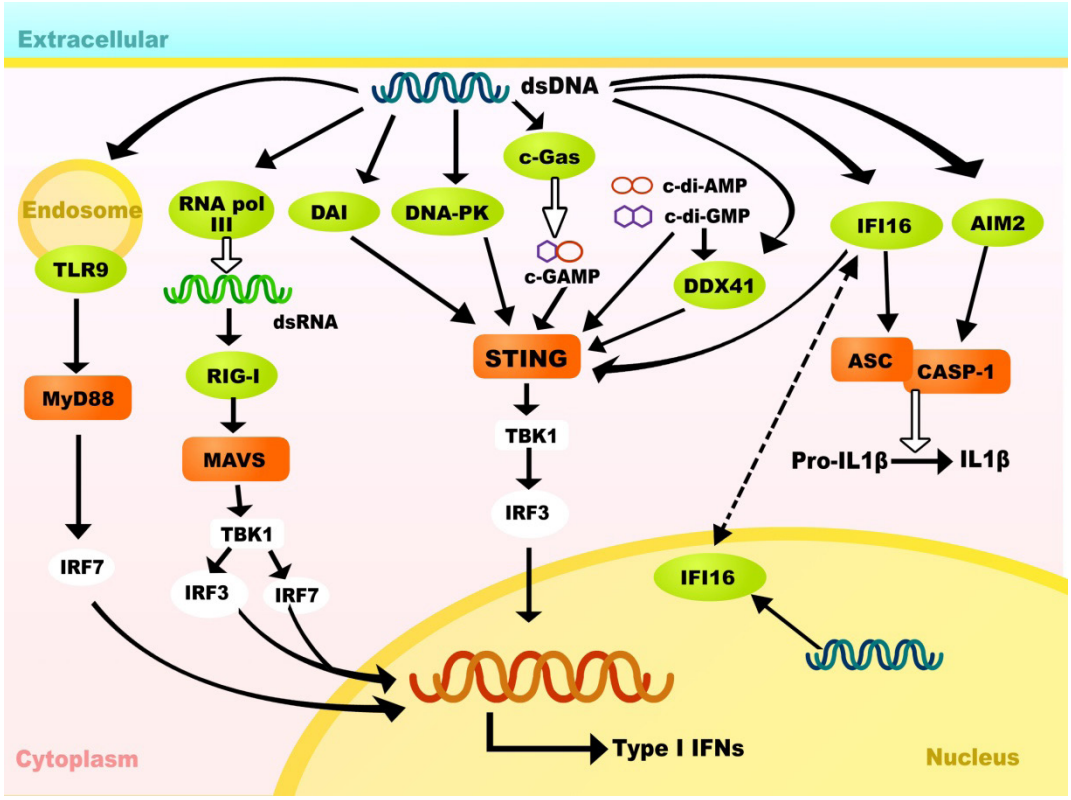


Figure I1. DNA sensors proposed and their signalling pathways. Simplified representation of several candidate DNA receptors and their main signalling pathways that activate type I IFN production and IL-1β maturation.

2. The PYHIN family

Both AIM2 and IFI16 proteins belong to a family of ISGs regulated by type I and II IFNs termed the **IFI200 family** (interferon inducible 200 family) or the **HIN-200 family**, which described their initially observed hematopoietic expression, IFN-inducibility, nuclear localization and characteristic 200 amino-acids repeat. This family comprises mammalian-specific proteins with similar domain structures, although it is not known whether they are true structural and functional orthologues, excluding AIM2 that is the only HIN-200 protein with orthology across various species (Deschamps, Meyer et al. 2003; Brunette, Young et al. 2012; Cridland, Curley et al. 2012). This family was first identified in mice (Kingsmore, Snoddy et al. 1989; Opdenakker, Snoddy et al. 1989) and the gene cluster is found in a syntenic region in many mammals, flanked by the *CADM3* (cell adhesion molecule 3) gene and by a set of olfactory receptors genes and the *SPTA1* (spectrin alpha, erythrocytic 1) gene, on chromosome 1q21-q23 in mouse and 1q23 in human (Brunette, Young et al. 2012; Cridland, Curley et al. 2012).

More recently, this family has also been called the PYHIN family because most of their members contain an amino-terminal PYRIN domain (known also as PYD, DAPIN or PAAD domain) and in most cases at least one carboxy-terminal HIN domain. The α-helical PYRIN domain, a member

of the death domain superfamily, is involved in homotypic protein-protein interactions associated with the regulation of apoptotic and inflammatory signalling pathways (Pawlowski, Pio et al. 2001; Staub, Dahl et al. 2001; Hiller, Kohl et al. 2003; Liepinsh, Barbals et al. 2003) while the HIN domain, a partially conserved repeat motif of 200-aminoacid residues, is involved in DNA binding and protein-protein interactions for transcriptional regulation (Koul, Obeyesekere et al. 1998; Johnstone, Kerry et al. 1998a; Johnstone, Wei et al. 2000; Yan, Dalal et al. 2008). The HIN domain is classified into three subtypes, designated as A, B and C, based on consensus motifs (Ludlow, Johnstone et al. 2005). Each 200-aminoacid repeat consists of two consecutive oligonucleotide/oligosaccharide binding (OB) folds (Albrecht, Choubey et al. 2005; Jin, Perry et al. 2012), commonly found in a number of single stranded DNA (ssDNA)-binding proteins (Liao, Lam et al. 2011).

The mouse family contains at least **fourteen members** (Brunette, Young et al. 2012; Cridland, Curley et al. 2012), among them **p202** and **p204** (Samanta, Engel et al. 1986; Choubey, Snoddy et al. 1989) (Figure I2) while in humans this family includes **five members: IFI16** (Trapani, Browne et al. 1992), **AIM2** (DeYoung, Ray et al. 1997), the Myeloid cell nuclear differentiation antigen (**MNDA**) (Burrus, Briggs et al. 1992), the Pyrin and HIN domain-containing protein 1 (PYHIN1, also called **IFIX**) (Ding, Wang et al. 2004) and the most recently identified Pyrin domain only protein 3 (POP3), which lacks the HIN domain found in other PYHIN proteins (Khare, Ratsimandresy et al. 2014) (Figure I2).

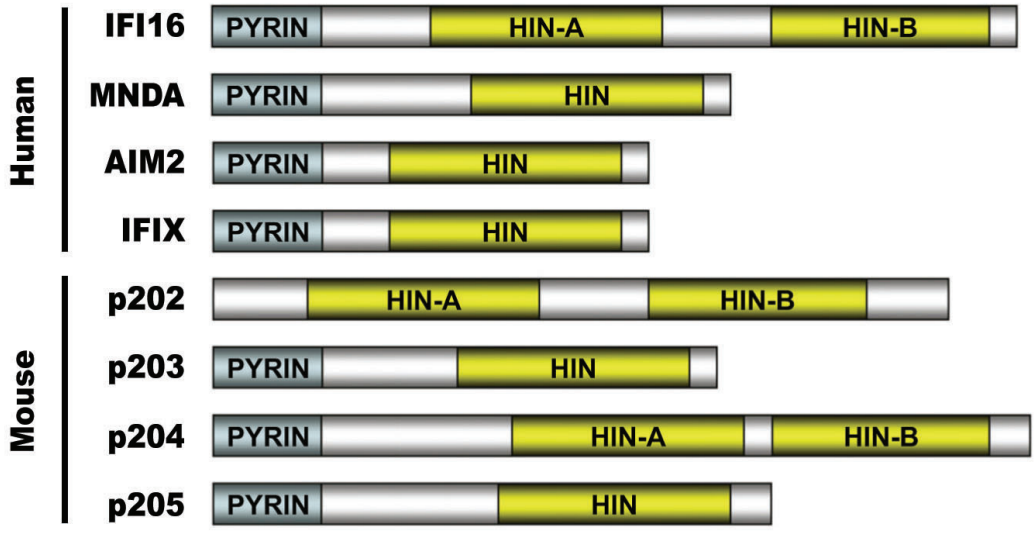


Figure I2. Domain organization of several human and mouse HIN-200 family members. *The Pyrin and HIN domains are indicated by grey and yellow boxes respectively. Adapted from Liao et al., 2011.*

3. The human gamma interferon-inducible protein 16 (IFI16)

General features

The *IFI16* gene was first described in 1992 by Trapani and colleagues. IFI16 mRNA was found to be constitutively expressed in lymphoid cells whereas the mRNA was not expressed in cells which represent early stages of myeloid development. (Trapani, Browne et al. 1992). However, treatment with IFNs α or γ strongly induced IFI16 mRNA and protein synthesis in myeloid cells (Trapani, Browne et al. 1992; Seelig, Ehrfeld et al. 1994).

Dawson and Trapani were also the first to propose that IFI16 was able to bind DNA (Dawson and Trapani 1995a) and they identified a DNA-binding site within the amino terminal 159 aminoacids (Dawson and Trapani 1995b). Therefore, IFI16 was clustered into the family of related human and mouse DNA-binding proteins known as the IFI200 family.

A particularity of IFI16 is the presence of **two HIN domains**, termed **HIN-A** and **HIN-B** separated by a serine-threonine-proline (S-T-P)-rich spacer region (hinge region). Moreover, **three spliced transcript variants** encoding different isoforms, clustered at 85-95 kDa, have been found. The nucleotide sequences of **IFI16 A**, **B**, and **C** (also called transcript variant 1, 2 and 3) are identical except for the region between the end of exon 6 and the start of exon 8. IFI16A contains exons 1 through 10 including exon 7a. The absence of exon 7a is characteristic of IFI16B, while IFI16C lacks both exons 7 and 7a (Johnstone, Kershaw et al. 1998b) (Figure I3). These splice variants encode proteins with different lengths of the hinge region, consequently changing the distance between the HIN A and B domains, and possibly changing the conformation of the molecule. However the functional significance, if any of the existence of these different proteins has not been studied. Notably, the B isoform is the predominant form detected in most cell types tested to date.

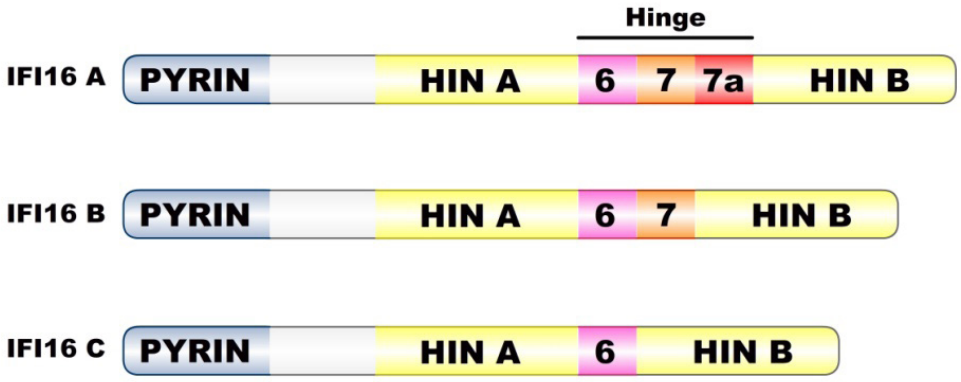


Figure I3. Schematic representation of the three different IFI16 isoforms arise due to alternative RNA splicing in the exons encoding the S/T/P region. Based on Johnstone et al., 1998b.

Tissue expression and subcellular localization

Since *IFI16* was first cloned, expression of this gene was considered specific for **hematopoietic cells**. Specifically, IFI16 has been detected in a wide variety of hematopoietic cells such as lymphocytes, specific myeloid progenitor cells (CD34+ progenitor cells) and monocytes. In contrast, its expression is down-regulated or not detected in other myeloid populations such as macrophages, granulocytes, erythrocytes and megakaryocites (Trapani, Browne et al. 1992; Dawson and Trapani 1995b; Dawson, Elwood et al. 1998). Nevertheless, IFI16 expression can be strongly induced in certain myeloid cell lines after exposure to IFN-γ, IFN-α (to a lesser extent) and chemical agents that drive differentiation along the myeloid lineage (Trapani, Browne et al. 1992; Dawson and Trapani 1995a).
However, in contrast to what was originally thought, IFI16 expression is not restricted to cells of the immune system since **it is also present in non-hematopoietic cells**, such as fibroblasts, endothelial and epithelial cells (Seelig, Ehrfeld et al. 1994; Gariglio, Azzimonti et al. 2002; Wei, Clarke et al. 2003).

IFI16 was originally considered as a strictly **nuclear protein** (Dawson and Trapani 1995a) due to the presence of a multipartite nuclear localization signal (NLS) (Dawson and Trapani 1995a; Briggs, Johnstone et al. 2001; Li, Diner et al. 2012a). In particular, although IFI16 was found in both nucleoli and nucleoplasm, it showed prominent nucleolar localization (Seelig, Ehrfeld et al. 1994; Dawson and Trapani 1995b). Indeed its expression was suggested to be restricted to transcriptionally active cells that have nucleoli (Dawson and Trapani 1995b). However, more recent studies have revealed that **IFI16 can also be found in the cytoplasm** in a variety of cell types (reviewed in (Veeranki and Choubey 2011)). Although several factors such as the presence of stimulatory DNA and the interactions of IFI16 with other proteins have been shown to influence the subcellular localization of IFI16 (reviewed in (Veeranki and Choubey 2011)), the acetylation of the IFI16 NLS has been defined as a key molecular mechanism that regulates IFI16 distribution. Specifically, acetylations at two critical sites within the NLS promote cytoplasmic localization by inhibiting nuclear import, and the acetyltransferase p300 has been identified as an enzyme involved in this process (Li, Diner et al. 2012a).

Biological Functions

Prior to the finding that IFI16 functions in pathogen recognition, studies focused on its roles in transcriptional regulation, cell differentiation, cell growth, senescence, apoptosis, tumour suppression, and autoimmunity.

As described previously, the levels of IFI16 vary with the differentiation state of the myeloid lineage. Moreover, it has been observed that IFI16 expression was induced with agents that promote myeloid differentiation (Dawson and Trapani 1995a). These first data suggested an association between IFI16 and **myeloid cell differentiation**.

Additionally, due to the double-strand (ds) DNA-binding capacity of IFI16 and its preferentially nuclear localization, early studies on IFI16 focused on its possible role in **transcriptional regulation** and **cell cycle control**. This hypothesis was reinforced by the previous findings that the related mouse protein p202 could interact with and modulate the transcriptional activities of several transcription factors including the retinoblastoma protein (pRb) (Choubey and Lengyel 1995), the E2 factor (E2F) family (Choubey, Li et al. 1996; Choubey and Gutterman 1997), the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), the activator protein-1 (AP-1) heterodimer comprising c-Fos and c-Jun proteins (Min, Ghosh et al. 1996) as well as p53 protein (indirectly via the interaction of p202 with the murine homolog of the human p53-binding protein (53BP1)) (Datta, Li et al. 1996).

IFI16 can also interact with several transcription factors, among which the members of the p53/Rb/E2F cell cycle regulatory axis are of particular importance for the antiproliferative activity of IFI16. Firstly, it was demonstrated that the N-terminal HIN-A domain of IFI16 bound the C-terminal region of p53, increasing p53-mediated transcriptional activity, and it was suggested that the interaction of IFI16 with p53 might be important for binding of p53 to DNA (Johnstone, Wei et al. 2000). More recently, it has been shown that the HIN-B domain of IFI16 also interacts with p53, specifically it recognizes the core domain of p53 stabilizing the p53-DNA binding (Liao, Lam et al. 2011). p53 functions as a transcriptional activator targeting key cellular genes such as the cell cycle kinase inhibitor p21 and the proapoptotic Bcl-2 (B-cell leukemia/lymphoma-2-alpha protein) family member Bax (Bcl-2-like-protein 4) among many others (reviewed in (Kruse and Gu 2009)). Knockdown of IFI16 expression in fibroblasts suppressed p53-mediated transcription resulting in down-regulated p21 protein levels and extended cell proliferation (Xin, Pereira-Smith et al. 2004). Moreover, IFI16 may also upregulate expression of p21 in a p53-independent manner (Xin, Curry et al. 2003). In addition to p21, IFI16 also enhanced the expression of the p53 target gene Bax (Fujiuchi, Aglipay et al. 2004).

IFI16 has also been implicated in the binding to both E2F1 and Rb (Xin, Curry et al. 2003). pRb suppresses cell cycle progression, in part, by binding to E2F transcription factors and inhibiting their transcriptional functions (reviewed in (Giacinti and Giordano 2006)). Thus, the binding of IFI16 to both proteins may potentiate the transcriptional repression of growth-promoting genes mediated by the binding of pRb to E2F (Xin, Curry et al. 2003) (Figure I4).

Further support for the involvement of IFI16 in p53 and Rb growth arrest pathways came from the finding that inactivation of both p53 and pRb by the E6 and E7 oncogenes of human papilloma virus (HPV) inhibited the cell growth suppression activity of IFI16 (Raffaella, Gioia et al. 2004).

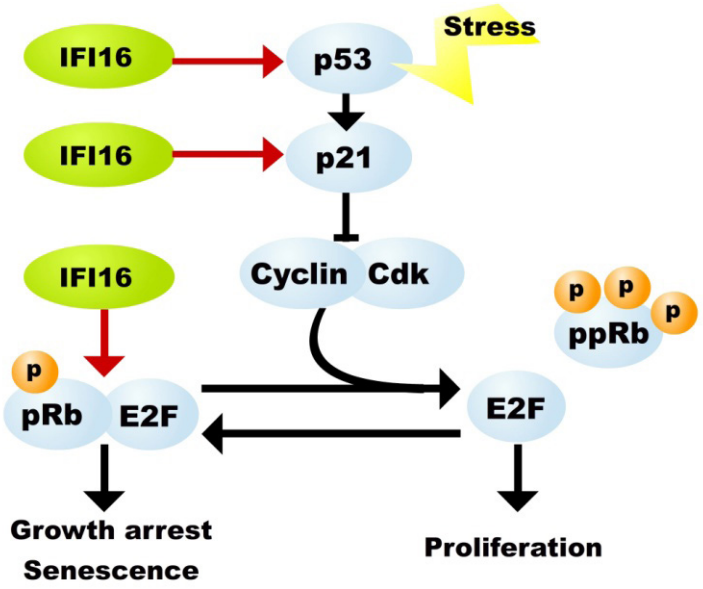


Figure I4. Schematic representation of cell cycle regulation by IFI16. After certain stress stimuli, the expression of cyclin-dependent kinase inhibitors such as p21 increases, mainly induced by p53, leading to an hypophosphorylated form of the retinoblastoma protein (pRb). In this state, pRb binds to E2F transcription factors inhibiting their activities, thereby suppressing cell growth. IFI16 can bind to pRb and E2F. IFI16 can also enhance the expression of p21 by interacting with p53 or in a p53-independent manner.

These results indicate that IFI16 generally collaborates with p53 to inhibit G1 to S phase transition. However, one report has suggested that IFI16 negatively regulates p53 because knockdown of IFI16 induced p53-dependent expression of p21 and reduced Rb phosphorylation leading to cell cycle arrest (Kwak, Ongusaha et al. 2003).

In addition to the ability of IFI16 to modulate the transcription function through the interaction with transcription factors, it seems that IFI16 can also directly regulate gene expression. Indeed, initially IFI16 was considered a potent transcriptional repressor and the 200-amino acids repeat regions were identified as the responsible of the repressive activity (Johnstone, Kerry et al. 1998a). Moreover, it has been shown that IFI16 can also interact directly with the gene promoters of p53 and c-myc (Egistelli, Chichiarelli et al. 2009).

All these pieces of evidence supported the idea that IFI16 could function as a transcriptional regulator, acting indirectly by modulating the activity of different transcription factors or even directly via the association of IFI16 with DNA on gene promoters.

In line with the ability of IFI16 to suppress cell growth, IFI16 can also regulate cellular **senescence** and **apoptosis**.

Bypassing the irreversible cell growth arrest associated with cellular senescence and becoming immortal is an early event in the malignant cell transformation. Among the key genes regulating

senescence/immortalization are the IFN-related genes. Specifically, the IFN-related pathways have been shown to be associated with cellular senescence (Fridman and Tainsky 2008). Consistent with a role of IFI16 in **cellular senescence**, IFI16 expression increased in normal human prostate epithelial cells as they approached cellular senescence. However, prostate cancer cell lines did not express IFI16. Furthermore, overexpression of IFI16 in human prostate cancer cells inhibited colony formation and resulted in a senescence-like phenotype associated with a reduction of cells in S-phase (Xin, Curry et al. 2003). Similarly, elevated levels of IFI16 were found in human "old" fibroblasts compared to "young" cells, and immortalization of fibroblasts resulted in diminished IFI16 expression (Xin, Pereira-Smith et al. 2004).

As mentioned before, a plausible mechanism by which IFI16 could inhibit proliferation and thus promote senescence would be the up-regulation of p21 expression and inhibition of E2F-stimulated transcription (Figure I4). Thus, loss of IFI16 function might contribute to bypass cellular senescence providing a proliferation advantage to cells.

As previously stated, IFI16 is also known to regulate **apoptosis**. IFI16 has been shown to bind BRCA1, a protein that plays a central role in several pathways that maintain genomic stability including DNA-damage response and DNA damage repair pathways (reviewed in (Wu, Lu et al. 2010)). The Pyrin domain of IFI16 binds to BRCA1 and this interaction has been reported to be involved in the p53-mediated cell death pathway in response to DNA damage, suggesting that IFI16 may play a role in DNA damage-induced apoptosis (Aglipay, Lee et al. 2003). Additionally, IFI16 is considered to a member of the multi-protein complex known as BASC (BRCA1-associated genome surveillance complex) (Aglipay, Lee et al. 2003), which contains tumor suppressors and DNA damage and DNA repair proteins (Wang, Cortez et al. 2000). These observations suggest that IFI16 could contribute to DNA damage and repair pathways.

Overall these data support the idea that IFI16 could play a role in tumor-suppression functions mediated by p53 and BRCA1 proteins, suggesting that loss of IFI16 function could result in deregulation of p53-mediated apoptosis, leading to cancer development. In accordance with the **tumor suppression activity** of IFI16, IFI16 expression is lost in several human tumours such as breast and prostate cancer cells (Xin, Curry et al. 2003; Fujiuchi, Aglipay et al. 2004).

The role of HIN-200 proteins, in particular IFI16, in regulating the processes of apoptosis and inflammation has also led to the suggestion that IFI16 could influence the development of **autoimmunity** (Gugliesi, De Andrea et al. 2010). Indeed, prior to the finding that IFI16 triggers inflammasome complex formation, it had been suggested that IFI16 could modulate inflammation through the induction of expression of proinflammatory molecules, and so it could play a role in the pathogenesis of autoimmune diseases (Caposio, Gugliesi et al. 2007). In addition, elevated levels of IFI16 autoantibodies were detected in serum samples from patients with different autoimmune

diseases including systemic lupus erythematosus (Seelig, Ehrfeld et al. 1994; Uchida, Akita et al. 2005; Mondini, Vidali et al. 2006), Sjögren's syndrome (Uchida, Akita et al. 2005; Mondini, Vidali et al. 2006) and systemic sclerosis (Mondini, Vidali et al. 2006). However, more studies are needed to better understand the role of IFI16 in autoimmunity.

As mentioned previously, although early studies focused on roles for IFI16 in transcriptional regulation, cell cycle control, cell differentiation and tumour suppression, more recently a role for IFI16 as an **innate immune sensor** of intracellular DNA has been identified (Unterholzner, Keating et al. 2010). Previous studies had provided evidences that other members of the PYHIN family, AIM2 and p202 proteins, could recognize DNA in the cytoplasm and upon sensing DNA the AIM2 protein formed and inflammasome leading to IL-1β maturation (Burckstummer, Baumann et al. 2009; Fernandes-Alnemri, Yu et al. 2009; Hornung, Ablasser et al. 2009). However, no PYHIN protein had been involved in type I IFN response to DNA until Unterholzner *et al.* identified IFI16 as a critical protein for the IFN-β response after transfection with cytoplasmic dsDNA derived from vaccinia virus or infection with herpes simplex virus type I (HSV-1) (Unterholzner, Keating et al. 2010). Later, IFI16 was also suggested to be involved in inflammasome assembly promoting proinflammatory cytokines maturation during Kaposi's sarcoma-associated herpesvirus (KSHV) (Kerur, Veettil et al. 2011).

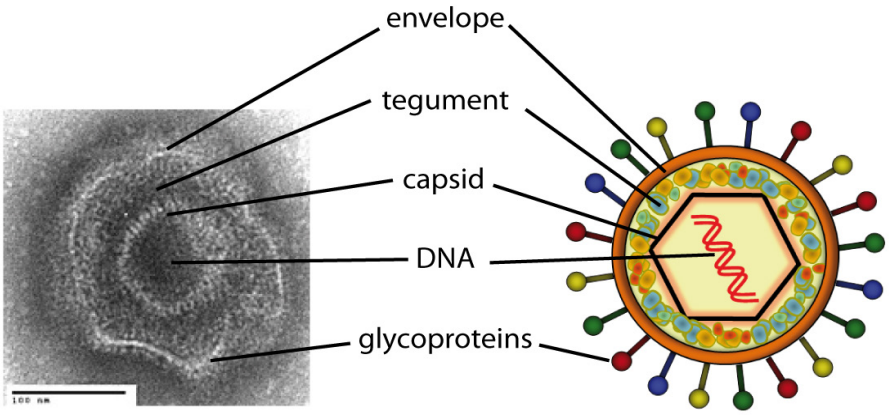
To date, it is known that IFI16 protein acts as a sensor for DNA during infection with a range of viruses including **HSV-1** (Unterholzner, Keating et al. 2010; Orzalli, DeLuca et al. 2012; Li, Diner et al. 2012a; Johnson, Chikoti et al. 2013), **KSHV** (Kerur, Veettil et al. 2011; Singh, Kerur et al. 2013), human cytomegalovirus (**HCMV**) (Cristea, Moorman et al. 2010; Li, Chen et al. 2013), Epstein-Barr virus (**EBV**) (Ansari, Singh et al. 2013), human immunodeficiency virus (**HIV**) (Jakobsen, Bak et al. 2013) and vaccinia virus (**VACV**) (Unterholzner, Keating et al. 2010) to induce the expression of **type I IFNs** and/or the maturation of proinflammatory cytokines such as **IL-1β**. These observations also revealed that IFI16 could sense pathogenic DNA in both the nucleus and the cytoplasm, being consistent with its dual subcellular localization.

In addition, IFI16 has been shown to exert a novel role in antiviral defense since it is involved in the **silencing of herpesvirus genomes**, via promoting the assembly of heterochromatin on HSV-1 genomic DNA (Orzalli, Conwell et al. 2013) and reducing the binding of various transcription factors to promoters of herpesviral genes (Gariano, Dell'Oste et al. 2012; Johnson, Bottero et al. 2014). Specifically, during HCMV infection IFI16 forms a stable complex with the transcriptional activator Sp1, therefore, displacing it from its promoters and consequently leading to a decrease in viral replication and gene expression (Gariano, Dell'Oste et al. 2012). IFI16 has also been found to be a restriction factor in HSV-1 infection by reducing the association of important transcription factors with promoters of several HSV-1 genes (Johnson, Bottero et al. 2014).

4. Herpes simplex virus type 1 (HSV-1)

The Herpesviridae family: A brief introduction

HSV-1 is a member of the *Herpesviridae* family, a group of large **DNA viruses** which contain linear, dsDNA. All herpesvirus share a common structure that consists of a core containing the **lineal ds-DNA genome**, an icosahedral **capsid** containing 162 capsomers, an amorphous layer of proteins that surround the capsid known as the **tegument**, and an **envelope** membrane containing viral glycoproteins spikes on its surface (Figure I5). The size of herpes virions varies from 120 nm to 300 nm. Herpesviruses are highly disseminated in nature infecting both vertebrates and nonvertebrates. All known herpesviruses promote **lytic infection** resulting in the production of infectious progeny virus accompanied by the destruction of the infected cell. They also have the unique ability to remain latent for the entire lifetimes of their hosts. In latency the viral genomes become circular and only a small subset of viral genes is expressed until some stimulus provokes the reactivation of the virus. The specific cell type in which they establish the **life-long latency** varies from one herpesvirus to another, but it primarily occurs in the ganglia of the nervous system and lymphoid tissue (Fields 1990).



(Dr. Colin Crump, University of Cambridge, UK)

Figure I5. Herpesvirus morphology. Virion structure of herpesviruses consisting of a core with the linear ds- DNA, an icosahedral capsid, the tegument and an envelope which contains the glycoproteins. **(Left panel)** Electron micrograph of a HSV-1 particle. All herpesviruses have identical morphology and cannot be distinguished from each other under electron microscopy. **(Right panel)** Schematic representation.

The members of the *Herpesviridae* family have been classified by the Herpesvirus Study Group of the International Committee on the Taxonomy of Viruses (ICTV) into three subfamilies- the **Alphaherpesvirinae**, the **Betaherpesvirinae** and the **Gammaherpesvirinae**- based on biological properties and genetic criteria (Roizman, Carmichael et al. 1981). To date, eight human herpesviruses have been described (Table I1).

Subfamily	Species
Alphaherpesvirinae	HHV-1, Herpes Simplex Virus type 1 (HSV-1)
	HHV-2, Herpes Simplex Virus type 2 (HSV-2)
	HHV-3, Varicella Zoster Virus (VSZ)
Betaherpesvirinae	HHV-5, Human Cytomegalovirus (HCMV)
	HHV-6
	HHV-7
Gammaherpesvirinae	HHV-4, Epstein Barr Virus (EBV)
	HHV-8, Kaposi's Sarcoma-associated herpesvirus (KSHV)

Table I1. Classification of human herpesviruses. HHV is the acronym for human herpesvirus.

HSV-1 (also called **human herpesvirus 1**, HHV-1) is one of the herpesvirus that specifically infects humans and belongs to the subfamily Alphaherpesvirinae and the genera Simplexvirus. There are two herpes simplex viruses, HSV-1 and HSV-2, which although very closely related by sequence homology and biological properties can be differentiated by a predilection for oral and genital mucosa respectively and the establishment of latency in the trigeminal and sacral ganglia respectively (Fields 1990).

Infection of human herpes simplex viruses is one of the oldest known orally and/or sexually transmitted infections and has been documented since ancient Greece. Hippocrates was the first to formally describe the spreading lesions that seem to be creeping or crawling through the skin (reviewed in (Nahmias and Dowdle 1968)). Indeed the word herpes comes from the Greek word "herpein" that means "to creep" (Beswick 1962). Shakespeare also described the recurrent labial lesions in "Romeo and Juliet" ("O'er ladies lips, who straight on kisses dream, which oft the angry Mab with blisters plagues, because their breaths with sweetmeats tainted are." Act 1. Scene IV) (noted by (Wildy 1973)).

HSV-1 is one of the **most common human pathogens worldwide**. Primary or initial infection occurs at mucosal and skin surfaces and is most often asymptomatic. While most diseases associated with primary and recurrent HSV-1 infections are relatively mild in the majority of patients (skin and lip lesions, gingivostomatitis, pharyngitis…), HSV-1 infection can occasionally cause severe and/or life-threatening diseases such as herpes simplex encephalitis, neonatal disseminated infection and blindness due to herpes simplex keratoconjunctivitis (Fields 1990).

Primary HSV-1 infection most often takes place at **oral mucosal and epithelial tissue**. Following replication of virus at portal of entry, the viral progeny infects the nerve termini of sensory neurons. The virions are carried by retrograde axonal transport to the neuronal cell bodies where they establish a **lifelong latency**, primarily in the **trigeminal ganglia**. Quiescent viral genomes can

occasionally reactivate into the lytic cycle due to a variety of stimuli, such as stress, fever and UV exposure. The new viral progeny travels by anterograde transport to the sites innervated by the infected neurons giving rise to recurrent infections (reviewed in (Diefenbach, Miranda-Saksena et al. 2008)).

An overview of the HSV-1 lytic replication cycle

To initiate infection, the virus must enter target cells. The initial attachment of the virion to the cell surface is followed by the fusion of the viral envelope with the plasma membrane and these events depend on the binding of certain viral glycoproteins to specific cell receptors. Briefly, virus attachment occurs via gC or gB binding to cell surface heparan sulfate proteoglycans. Following this initial attachment, gD interacts with one of its cellular entry receptors: nectin-1, herpesvirus entry mediator (HVEM) or 3-O-sulfated heparan sulfate. After this interaction, gD undergoes conformational changes that activate the interaction of gB and the heterodimer gH-gL with the cellular membrane leading to membranes fusion (reviewed in (Reske, Pollara et al. 2007)). Fusion of the viral membrane with the host membrane releases into the cytoplasm the majority of tegument proteins including vhs (virion host shutoff protein) and α-TIF (α gene trans-inducing factor, also called Vmw65 or VP16). vhs triggers inhibition of host cell protein synthesis, disruption of preexisting polyribosomes and degradation of host mRNAs before the onset of de novo viral gene expression. This increases the efficiency of viral gene expression (reviewed in (Smiley, Elgadi et al. 2001)). VP16 interacts with the host cell factor-1 (HCF-1), which is involved in the transport of VP16 to the nucleus (La Boissiere, Hughes et al. 1999). Following fusion of the membranes the viral capsid is also released to the cytoplasm and rapidly transported via microtubules to the nuclear pore complexes (Sodeik, Ebersold et al. 1997), where viral DNA is released into the nucleoplasm. The linear HSV-1 DNA is circularized (Garber, Beverley et al. 1993; Strang and Stow 2005), serving as a template for transcription and replication of the viral genome.

HSV-1 gene expression is organised into a temporally regulated cascade, where three temporal classes of viral genes are sequentially expressed: immediate early (IE or α), early (E or β) and late (L or γ) genes (Honess and Roizman 1974). The five IE genes are the first to be expressed (known as infected cell polypeptides ICP0, ICP4, ICP22, ICP27 and ICP47). The transcription of these IE genes by cellular proteins is stimulated by the VP16 protein (Campbell, Palfreyman et al. 1984). The VP16/HCF-1 complex induces a trimeric DNA binding complex by interacting with the cellular Octamer-binding protein 1 (Oct-1) on regulatory elements located upstream of the IE genes promoters (reviewed in (Wysocka and Herr 2003)). All the IE proteins except ICP47, which blocks viral peptide loading onto nascent MHC (major histocompatibility complex) class I molecules by binding to TAP (transporter associated with antigen processing) (York, Roop et al. 1994; Hill, Jugovic et al. 1995), are involved in the regulation of viral gene expression both transcriptionally and posttranscriptionally. Briefly, ICP4 is the major transactivator of gene expression and is necessary for transcription of both E and L genes. ICP27 is also an essential gene, which is responsible for L gene expression and normal expression

levels of several E genes. It predominantly functions at a post-transcriptional manner through mRNA processing. ICP0 is not absolutely essential for productive infection in cell culture systems; however, ICP0-deficient mutants are severely impaired for viral growth, particularly at low multiplicities of infection. It has been considered a promiscuous transactivator of gene expression due to its ability to stimulate expression of IE, E and L genes, as well as several cellular genes in transient transfection assays. ICP0 also induces the degradation of a number of cellular proteins. ICP22 is not an essential gene for viral growth and viability in most cell types and is required for optimal expression of a subset of L genes (reviewed in (Weir 2001)). Therefore, IE gene expression leads to the expression of E genes, the "leaky-late" (L1 or γ-1) genes prior to viral DNA replication, and finally the "true-late" (L2 or γ-2) at the onset of DNA replication. Generally, the E genes encode proteins required for viral DNA replication and the L genes give rise to the structural proteins.

Given that the virus encodes its own replication machinery, the viral DNA strands are synthesized by viral DNA polymerase (UL30) complexed with the processivity factor UL42. In addition to the viral proteins necessary for viral DNA synthesis, several cellular proteins take part in viral replication (reviewed in (Weller and Coen 2012)). As mentioned, viral DNA replication begins prior to the expression of L genes. After an initial phase of theta replication, viral DNA replication switches to a rolling-circle mechanism generating concatemers that are cleaved into monomers and packaged into preassembled capsids (Skaliter and Lehman 1994).

After the capsid assembly and viral genome packaging in the nucleus, the nucleocapsids have to cross the nuclear envelope in order to complete their maduration in the cytosol. A model largely accepted comprises the budding of the nucleocapsids at the inner nuclear membrane resulting in the formation of a primary envelope. Then, this envelope fuses with the outer nuclear membrane releasing the maturing capsids into the cytoplasm (reviewed in (Mettenleiter, Muller et al. 2013)). Following nuclear egress, most of the tegument proteins are added to the capsids. Moreover, the capsids acquire the final envelope and surface glycoproteins by budding into trans-Golgi vesicles. Final mature virions are released by fusion of the vesicles with the plasma cell membrane (reviewed in (Mettenleiter, Klupp et al. 2009)).

ICP0 modulation of host cell responses to HSV-1 infection

Viruses are obligate intracellular parasites that provoke complex responses from the infected host cells, which have developed numerous mechanisms in order to restrict viral infections. However, during the course of coevolution with their hosts, viruses have evolved varied mechanisms to circumvent the deleterious effects of the cellular pathways or exploit these signaling pathways for their own gain. Viral neutralisation of host antiviral responses relies on the ability to mimic structure or function of cellular proteins. For example several viruses, including HSV-1, encode proteins that are similar to cellular E3 ubiquitin ligases. A particular focus of the work presented in this thesis is the **HSV-1 E3**

ubiquitin ligase ICP0.

As mentioned before, ICP0 expression begins during the initial stages of infection. Although it is not absolutely essential for productive lytic infection, it is required for efficient viral replication since ICP0-null mutants are severely impaired for viral growth, particularly at low multiplicities of infection (reviewed in (Weir 2001)). It also stimulates the reactivation of latent viral genomes. Therefore, it plays a **key role in regulating both lytic and latent infections**. These requirements of ICP0 have been linked to its ability to stimulate gene expression from a wide range of viral promoters. ICP0 has also been suggested to regulate multiple other aspects of the cellular response to viral infection including protein stability, chromatin modification, the intrinsic antiviral resistance, the IFN response and the DNA damage response (DDR) pathways (reviewed in (Boutell and Everett 2013; Lanfranca, Mostafa et al. 2014)).

ICP0 contains an N-terminal **RING finger domain** that confers **E3 ubiquitin ligase activity**, which mediates ubiquitination and therefore proteasome-dependent degradation of a number of cellular proteins during infection. Basically the E3 ubiquitin ligases interact with both E2 ubiquitin-conjugating enzymes and substrate proteins mediating the transfer of ubiquitin from E2 enzymes to the substrates (reviewed in (Deshaies and Joazeiro 2009)). Ubiquitination in turn targets the substrate for degradation by the proteasome, although this modification can also have other effects on the targeted protein. ICP0 uses diverse mechanisms to target different cellular proteins. It can interact directly with the substrate through specific recognition motifs or in a phosphorylation-dependent manner. On the other hand, it can target other substrates in a SUMO (small ubiquitin-like modifier)-dependent way or indirectly via binding partner(s) (reviewed in (Boutell and Everett 2013)

The RING finger domain of ICP0 plays a fundamental role in the biology of ICP0 since mutations resulting in defective RING finger domain not only impaired its ubiquitin ligase activity but also resulted in replication defects equivalent to that of ICP0-null mutants and failed de-repression of latent HSV-1 genomes (reviewed in (Boutell and Everett 2013)

The **targets of ICP0** for proteasome-dependent degradation include different proteins, many of which are implicated in **host cellular defences** that restrict viral infection (reviewed in (Boutell and Everett 2013; Lanfranca, Mostafa et al. 2014)). This indicates the dynamic nature of the interactions of ICP0 with cellular components during the infection, a few of which are described in more detail below.

PML (promyelocytic leukemia protein) is one of the best characterized substrates of ICP0. It is a principal component of nuclear domains 10 (**ND10,** also known as PML nuclear bodies), which are accumulations of several proteins important for repressing viral replication and transcription (reviewed in (Tavalai and Stamminger 2008)). These proteins participate in intrinsic immunity, which is a form of innate immunity composed of constitutively expressed proteins ready to immediately and directly repress viral infection (reviewed in (Bieniasz 2004; Yan and Chen 2012)). In addition to PML, other ND10 members include **Sp100** (speckled protein 100kDa), **hDaxx** (human death domain-associated protein 6) and **ATRX** (ATP-dependent helicase alpha thalassemia/mental retardation syndrome X-linked). ND10 components have been shown to relocate to sites associated with incoming HSV-1

genomes during initial stages of infection (Everett and Murray 2005). However, **ICP0 promotes the disruption of ND10 by inhibiting the recruitment of PML, Sp100, hDaxx, ATRX and the SUMO family of proteins** (Maul, Guldner et al. 1993; Everett and Maul 1994; Lukashchuk and Everett 2010; Cuchet-Lourenco, Boutell et al. 2011) **as well as inducing the degradation of PML** (Everett, Freemont et al. 1998; Chelbi-Alix and de The 1999); thereby counteracting this type of intrinsic antiviral defense. Another part of the intrinsic cellular defense against HSV-1 infection depends on proteins of the **DNA damage response** (DDR) (reviewed in (Weitzman, Lilley et al. 2010)). The DDR is a complex network of cellular pathways which detect, signal and repair DNA lesions in order to maintain the genomic stability and prevent the transmission of genetic mutations. The presence of a DNA lesion is detected by sensor proteins that activate signalling pathways leading to a broad range of cellular responses (reviewed in (Jackson and Bartek 2009)).

The primary signalling pathway mediators that initiate DDR are the kinases **ATM** (ataxia-telangiectasia mutated), **ATR** (ATM and Rad3-related protein) and **DNA-PK** (DNA-dependent protein kinase). While ATM and DNA-PK are activated in response to dsDNA breaks, ATR is recruited to ssDNA breaks.

In the case of ATM-dependent signalling pathway, dsDNA breaks are recognized by the cellular **Mre11-Rad50-Nbs1 (MRN) complex**, which activates the **ATM** (ataxia-telangiectasia mutated) kinase resulting in the phosphorylation of histone **H2AX** on the chromatin surrounding the break. The mediator protein **Mdc1** (mediator of DNA damage checkpoint protein 1), which is anchored to the phosphorylated H2AX (γ-H2AX), recruits the downstream factor **RNF8** (RING finger protein 8). Then RNF8, which is an E3 ubiquitin ligase, ubiquitinates proteins at the site of the break including the histones H2A and H2AX. These ubiquitin chains in turn promote the recruitment of **RNF168** (RING finger protein 168), another E3 ubiquitin ligase. These sets of ubiquitination events orchestrate the recruitment of downstream proteins such as **53BP1** (p53 binding protein 1) and **BRCA1**. Finally, effector proteins including **chk2** (checkpoint kinase 2) and **p53** trigger the cellular response to DNA damage such as cell cycle modulation, apoptosis and DNA repair (reviewed in (Weitzman, Lilley et al. 2010)).

DNA-PK plays a central role in dsDNA break repair via non-homologous end-joining (NHEJ) activity. DNA-PK comprises a large catalytic subunit (**DNA-PKcs**) and two regulatory subunits (**Ku70** and **Ku86**). The Ku complex binds to the broken DNA and then recruits DNA-PKcs which interacts with the DNA (reviewed in (Jette and Lees-Miller 2015)).

Many viruses including HSV-1 activate the same cellular cascades induced by DNA damage. The DDR factors may recognize the incoming viral genomes as damaged DNA and provide a defense against these pathogens. In turn, viruses have evolved ways to manipulate the DDR repressing or activating it to promote their own replication (reviewed in (Weitzman, Lilley et al. 2010; Turnell and Grand 2012)). In the case of HSV-1, it has a complex relationship with the DDR pathways (reviewed in Turnell 2012). For example, although HSV-1 infection induces the activation of ATM and the subsequent activation of the downstream targets including chk2 and p53 (Shirata, Kudoh et al. 2005;

Li, Baskaran et al. 2008), the ATM-dependent signaling pathway is only partially activated since **ICP0 has been shown to induce the degradation of RNF8 and RNF168** (Lilley, Chaurushiya et al. 2010). This could prevent the deposition of repressive ubiquitin marks and hence counteract the transcriptional silencing of the viral genomes (Lilley, Chaurushiya et al. 2011).

In addition to RNF8 and RNF168, **ICP0 has also been shown to mediate the degradation of the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs)** (Lees-Miller, Long et al. 1996; Parkinson, Lees-Miller et al. 1999), thereby counteracting the NHEJ repair function of DNA-PK. Additionally, DNA-PK has been shown to act as a DNA sensor that leads to the production of type I IFN, cytokines and chemokines in an IRF-3-dependent manner during infection with DNA viruses, such as HSV-1 (Ferguson, Mansur et al. 2012). Thus, it is possible that ICP0 could also act to counter this innate immune response.

As well as "intrinsic antiviral factors", other innate immune factors are also activated during viral infections via the detection of viral components that leads to the induction of host cytokines, such as type I IFNs. Type I IFNs trigger the expression of ISGs, which establish an antiviral state in cells (reviewed in (Ivashkiv and Donlin 2014)). ICP0 has been shown to counteract the activation of the type I IFN response by targeting several regulatory factors including IFI16.

As already stated, as well as viral DNA sensor that triggers the expression of type I IFN or proinflammatory cytokines, IFI16 exerts "intrinsic" antiviral activity by silencing HSV-1 genome and hence inhibiting viral gene expression. Therefore, it is not surprising that **IFI16 is targeted for degradation by ICP0**, presumably in response to evolutionary pressures to counteract the antiviral functions of IFI16.

The first evidence for IFI16 degradation promoted by ICP0 was presented by Orzalli et al., who observed that shortly after infection of human foreskin fibroblasts (HFF) with a mutant HSV-1 virus which expresses ICP0, but not additional IE gene products, nuclear IFI16 was partially localized with ICP0 and its expression was markedly reduced by 4-6 hours post infection. They also observed that treatment of HFFs with a proteasome inhibitor inhibited the loss of IFI16 observed during infection with wild type (wt) virus, and an ICP0 RING finger mutant virus was not able to trigger the loss of IFI16. Thus, they concluded that ICP0 promoted the IFI16 degradation in a proteasome- and RING finger domain-dependent mechanism (Orzalli, DeLuca et al. 2012).

These observations were confirmed and extended by subsequent work (Johnson, Chikoti et al. 2013). More recently, however, conflicting data have been presented by Cuchet-Lourenço et al., who indicated that ICP0 is neither sufficient nor necessary for the degradation of IFI16 during the course of HSV-1 infection and suggested that the effects of ICP0 on IFI16 degradation were indirect and related to the role of ICP0 in modulating the progression of viral infection (Cuchet-Lourenco, Anderson et al. 2013).

The findings of Cuchet-Lourenço *et al*. demand a reassessment of the changes in IFI16 levels during HSV-1 infection. We have approached this problem using HSV-1 mutants that express ICP0 but from which the ICP4 and the rest of IE genes have been deleted. These viruses are unable to complete

a normal cycle of viral replication due to the absence of the major transcriptional activator ICP4. Therefore, infection with these viruses allows the effect of ICP0 to be distinguished from those of the progression of viral infection. This work is the main subject of the section 1 of Results.

Furthermore, during these experiments a possible effect of HSV-1 infection on BRCA1 expression was studied and a detailed exploration of these experiments is presented in the Section 2 of Results. The progress of these experiments also involved other aspects of the biology of IFI16 including the expression of this receptor in a range of tumor cell lines and these data are presented in the Section 3.

Objectives

The main objectives addressed in the experimental work of this thesis were the following:

1. Analyse the effect of HSV-1 infection on IFI16 expression and study the role of ICP0 in IFI16 destabilization.

2. Examine the effect of HSV-1 infection on the expression of IFI16-interacting proteins such as p53 and BRCA1.

3. Identify the 120 kDa protein, recognised by a BRCA1-reactive antibody, upregulated after HSV-1 infection.

4. Analyse the expression of IFI16 protein in a range of tumour cell lines.

Materials and Methods

Cells

Telomerase-immortalized human foreskin fibroblast (HFF) cells (McSharry, Jones et al. 2001), (a gift from Gavin Wilkinson), the Vero cell line, derived from kidney epithelial cells of an African green monkey, the human osteosarcoma (U2OS) and baby hamster kidney (BHK) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM). 49-44 cells (a complementing cell line derived by transfection of Vero cells to express gH, ICP4, and ICP27) were grown in DMEM with 0.6 mg/mL G418 and 4 μg/mL puromycin. The cell line U2OS stably expressing GFP-BRCA1 and FLAG-BARD1 (a gift from Jackson's lab, The Gurdon Institute, University of Cambridge, UK) were cultivated in DMEM with 0.6 mg/mL G418.

The human colorectal carcinoma (HCT116) cell line expressing wild-type p53 and the HCT116 cell line with both p53 alleles inactivated by homologous recombination (Bunz, Dutriaux et al. 1998) were provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) and cultured in Roswell Park Memorial Institute (RPMI) medium.

The cellular pellets from the breast cell lines MCF10A, MDA-MB-468, BT-474, SK-BR-3, MDA-MB-361, MDA-MB-453, T-47D, Hs578T and BT-549 were provided by Dr. Pedro Torres Ayuso (National Centre for Biotechnology, Madrid). The HB4a cell line was a gift from Dr. Paul Edwards (Stamps, Davies et al. 1994) (Department of Pathology, University of Cambridge, UK). The breast cancer cell lines MCF7 and MDA-MB-231 (a gift from Dr. Lourdes Planelles, National Centre for Biotechnology, Madrid) were cultivated in Minimum Essential Medium (MEM) and DMEM respectively.

The cellular pellets from all the bladder cancer cell lines (RT4, J82, UM-UC-3, T24 and RT-112) were provided by Eva Mª García Cuesta from the group of Dr. Mar Valés (National Centre for Biotechnology, Madrid).

All cell lines were maintained at 37°C and 5% CO2 in a humidified incubator. All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2mM L-Glutamine, 0.1mM non essential amino-acids, 10mM HEPES, 1mM sodium pyruvate, 100U/mL penicillin, 100U/mL streptomycin and 50μm β-mercaptoethanol.

Viruses

The HSV-1 SC16 ΔgE, *in*1814, tsK, *in*1382 and HSV-2 viruses were provided by Dr. Stacey Efstathiou's group (Division of Virology, Department of Pathology, University of Cambridge, UK).
The dh1 series of HSVs (Chisholm, Howard et al. 2007), derived from gH-null HSV-1 strain 17 (Forrester, Farrell et al. 1992) were gifts from Xenova Research. The HSV-1 *dl*1403 and the control revertant *dl*1403R (Stow and Stow 1986) were gifts from Dr. R. Everett and Dr. N. Stow (MRC Virology Unit, Institute of Virology, Glasgow, UK).

VACV (strain WR) and HCMV (strain AD169) were provided by Dr. Antonio Alcamí (CBMSO, CSIC) and Drs. Helena Browne and Tony Minson (Division of Virology, Department of Pathology,

University of Cambridge, UK) respectively.

Production of virus stocks

Cell-associated virus stocks (Standard virus stocks)

Confluent BHK cells in 150 cm2 flasks were infected with virus stocks at 0.01 pfu/cell in a small volume of culture media and incubated for 1h at 37°C in constant rocking to allow the virus adsorb and enter. After which cells were overlaid with more volume of media and incubated at 37°C for 2-4 days. Cells were harvested by scraping the culture flasks and pelleted by centrifugation (1400 rpm 5 min RT). The cell pellet was resuspended in a small volume of culture media (1-3 ml) and sonicated at 50% amplitude for 2 min aprox. before being divided into aliquots and stored at -70°C. Viral titres were then determined by plaque assay.

To produce the stock of *in*1814 was necessary to supplement the media with N,N'-hexamethylene bisacetamide 98% (Sigma Aldrich). This compound stimulates IE gene expression and thus enables *in*1814 to initiate productive infection (McFarlane, Daksis et al. 1992).
Stocks of *dl*1403 and *dl*1403R viruses were prepared in Vero cells and the dh1a, dh1b, dh1c and dh1f stocks were prepared in 49-44 cells.

Purified virus stocks

The procedure was started as for standard virus stocks, however, when cells were pelleted by low speed centrifugation, the resultant supernatant was collected and subjected to high-speed centrifugation (18000 rpm 2h 4°C) to recover the virions from the supernatant. Viral pellets were resuspended in small volume of culture media (2-3 ml), sonicated as described above and aliquoted for storage at -70°C. Viral titres were then determined by plaque assay.

Plaque assay by toluidine blue staining

Titers of infectious virus were determined by plaque assay on BHK cells. BHK cells were grown in 6-well plates and 10x serial dilutions in serum-free DMEM of viral stocks were incubated on BHK monolayers for 1h at 37°C. The inoculum was overlaid with DMEM containing 0.3% carboxymethylcellulose. After 2-3 days of incubation at 37°C, the inoculum was aspirated and monolayers were fixed in 4% formaldehyde for 30 min and stained with 0.1% toluidine blue for 30 min. Plaques were counted using a plate microscope.
Stocks of *dl*1403 and *dl*1403R viruses were titered in U2OS cells and the dh1a, dh1b, dh1c and dh1f stocks were titered in 49-44 cells.

Infections

Cells were mock-infected or infected at the indicated m.o.i. for 2 h at 37°C in serum free DMEM, incubated overnight in DMEM supplemented with 10% with FBS and washed with phosphate-buffered

saline (PBS) before harvesting and lysis in RIPA lysis buffer (50mM Tris-Cl pH 7.4, 150mM NaCl, 1% Triton X-100, 1% Deoxycholate, 0,1% sodium dodecyl sulfate (SDS)) in the presence of protease inhibitors (1μg/ml Pepstatin A, 1μg/ml Leupeptin, 1mM Iodoacetamide, 10mM 1, 10-Phenanthroline) and phosphatase inhibitors (0,02M Sodium Fluoride (NaF) and 2mM Sodium Orthovanadate (Na3VO4)) and 1μg/ml DNase for 30 minutes on ice. After centrifugation at 14000 rpm for 15 minutes at 4°C to remove debris, supernatants were collected as cell lysates.

During the stay at the University of Cambridge the cells were mock-infected or infected in DMEM supplemented with 10% fetal calf serum (FCS) and incubated overnight prior the lysis in lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 5mM EDTA , 1mM phenylmethylsulfonyl fluoride (PMSF)) supplemented with protease inhibitor cocktail (Roche).

Western blot analysis.

Cells were lysed in RIPA lysis buffer (50mM Tris-Cl pH 7.4, 150mM NaCl, 1% Triton X-100, 1% Deoxycholate, 0,1% SDS) with protease inhibitors (1μg/ml Pepstatin A, 1μg/ml Leupeptin, 1mM Iodoacetamide, 10mM 1, 10-Phenanthroline) and phosphatase inhibitors (0,02M NaF and 2mM Na3VO4) and 1μg/ml DNase for 30 minutes on ice. Lysates were then centrifugated at 14000 rpm for 15 min at 4°C to pellet nuclei and remove debris. Supernatants were collected as cell lysates and protein concentration was quantified using the Coomassie protein assay reagent (Thermo Scientific). 2x loading buffer (125mM Tris pH 6.8, 4% SDS, 20% glycerol and bromophenol blue) with 100mM dithiotreitol (DTT) (Melford Laboratories) was added to the indicated μg of the proteins lysates and then the samples were heated 100°C for 5 min and resolved by SDS-Polyacrylamide gel electrophoresis (PAGE) on either 8% or 10% resolving gels at 200 V for 1h aprox. (Electrophoresis running buffer: 195 mM Glycine, 24.8 mM Tris Base, 1% w/v SDS). Then the proteins were transferred to activated polyvinylidene fluoride (PVDF) membranes (Immobilon-P Millipore). The PVDF membrane was placed underneath the gel flanked by thick filter paper (BioRad), all soaked in transfer buffer (192 mM Glycine, 25mM Tris base, 20% methanol and 0.03 % w/v SDS), and proteins were transferred using a Trans-Blot® Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) in accordance with the manufacturer's instructions. The membrane was blocked using 5% skimmed milk in TBS (150mM NaCl, 50mM Tris-Cl pH7.5) with 0.05% Tween-20 (TBS-T) for 1h at room temperature. After washing three times using TBS-T (10 min per wash), the membrane was incubated with the corresponding primary antibody diluted in TBS-T overnight (o/n) at 4°C, washed again with TBS-T and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Dako) diluted in TBS-T for 1h at room temperature. The membrane was washed and bound primary antibody was visualized by reaction with Amersham™ ECL™ Western Blotting Analysis System (GE Healthcare) and exposure to x-ray films (Konica Minolta and Agfa).

For the experiments carried out at the University of Cambridge, this protocol was varied slightly

(Figs. R12-R17, R33-R36). Cells were lysed in lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 5mM EDTA, 1mM PMSF) supplemented with protease inhibitor cocktail (Roche). Primary and secondary antibodies were incubated with 5% skimmed milk in TBS-T. Primary antibody was incubated o/n unless otherwise indicated. The incubation with the secondary antibody was performed in the dark due to its light sensitivity. And the bound primary antibody was detected using an Odyssey infra-red imaging system (LICOR Biosciences).

Cell fractionation and preparation of nuclear extracts

Cell fractionation was performed as described previously (Meyer and Radsak 2000). Cells pellets were resedimented in TKM buffer (25 mM Tris-HCl pH 7.4, 5 mM KCl, 1 mM MgCl2) and resuspended in 0.5 ml of the same buffer (5 min 4°C). Then 0.5 ml of TKM buffer containing 1% Nonidet P-40 (NP-40) was added and the mixture was incubated (5 min 4°C) with occasional vortexing. Nuclei were sedimented (1000 x g 5 min 4°C) and resuspended in TKM buffer containing 0.5% NP-40 and 100 mM NaCl. The supernatant fraction was saved as the postnuclear fraction (called cytosolic fraction in this thesis). Nuclei were separated from inner membranes by centrifugation (2100 x g 15min 4°C) through a 3 ml cushion of 1.62 M sucrose in TKM buffer. The pelleted nuclei were washed with TKM buffer containing 0.5% NP-40 and 100 mM NaCl. The postnuclear fraction and the nuclear pellet were lysed in RIPA buffer prior to SDS-PAGE and western blot analysis.

Immunoprecipitation

Cells were lysed in lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 5mM EDTA, 1mM PMSF or 10 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1mM PMSF, 10% glycerol, 1 mM Na3VO4, 1 mM NaF) with protease inhibitors cocktail (Roche) for 20 min on ice. Insoluble debris was removed by centrifugation (14000 rpm, 10 min 4°C) and cell lysates were incubated with 2μg of the specific antibody o/n at 4°C on a rotating wheel followed by addition of pre-cleaned Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) beads for 2h at 4°C with constant rotation (beads were previously cleaned by washing and resuspending the Protein A/G PLUS-Agarose beads in lysis buffer). Beads were pelleted by centrifugation (13000 rpm 30 s) and washed 2x in the ice-cold lysis buffer. Beads were then repelleted by centrifugation (13000 rpm 1 min) and 40 μl of 2x SDS-PAGE loading buffer were added to bead samples and boiled for 5 min to elute the immunoprecipitated proteins from the beads prior to SDS-PAGE analysis.

Primary antibodies	Supplier
IFI16 (1G7)	Santa Cruz Biotechnology
ICP0 (11060)	A gift of Dr. Roger Everett
ICP4 (58S)	A gift of Dr. Roger Everett
VP16 (1246)	A gift of Dr. Enrique Tabares
gD (r27)	A gift of Dr. Enrique Tabares
UL42 (13C9)	Santa Cruz Biotechnology
p53 (DO-1)	Santa Cruz Biotechnology
BRCA1 (I-20)	Santa Cruz Biotechnology
gD (LP14)	A gift of Dr. Colin Crump
BRCA1 (D-20)	Santa Cruz Biotechnology
EGFR	A gift of Dr. Pedro Torres
GFP (ab290)	Abcam
GFP (JL8)	A gift of Dr. Colin Crump
UL37	A gift of Dr. Colin Crump
LaminA/C (636)	Santa Cruz Biotechnology
MEK1/2 (47E6)	Cell Signaling
β-actin AC-15	Sigma-Aldrich
β-actin (AC-40)	Sigma-Aldrich
Secondary antibodies	**Supplier**
Goat anti-rabbit HRP	Dako
Goat anti-mouse HRP	Dako
IRDye 800CW donkey anti-mouse/rabbit	LICOR Bioscience
IRDye 680 donkey anti-mouse/rabbit	LICOR Bioscience

Table M1. Primary and secondary antibodies used for immunoprecipitation and western blot analyses. The commercial source or the academic lab that provided the antibody are listed.

Coomassie staining of SDS-PAGE gels

The immunoprecipitated proteins separated by SDS-PAGE were stained with Coomassie Blue dye. After electrophoresis, gels were incubated with 0.1% (w/v) Coomassie Brilliant Blue R-250 in fixing solution (40% methanol and 10% acetic acid) for 60 min at RT. The staining solution was removed and gels were incubated with destain solution I (50% methanol) for 30 min at RT and destain solution II (40% methanol, 10% acetic acid) at RT for 1-2h or until the background staining had been removed.

Mass spectrometry

The proteins visualized by Coomassie blue stain were identified by Peptide Mass Fingerprinting. Basically, the protein bands visualized by Coomassie stain were excised from the gel and the proteins in the excised gel band were digested using trypsin. The resulting peptides were analyzed by MALDI/TOF mass spectrometry (MS). MS analysis was completed by Cambridge Centre for Proteomics Core Services (http://www3.bioc.cam.ac.uk/paservices.html). The identification of each protein was determined using Mascot software.

Off Gel analysis was performed by the Proteomics Facility of the Spanish National Centre for Biotechnology.

Immunofluorescence and confocal microscopy

Cells grown on glass coverslips were subjected to virus infection or not. After the appropriate time, the media was discarded and cells were washed with PBS (138mM NaCl, 2.7mM KCl, 8mM Na2HPO4, 1.5mM KH2HPO4 pH 7.6) and fixed in 4% paraformaldehyde for 20 min at room temperature. Then cells were washed with PBS-0.1% Tween (PBS-T) and permeabilised with PBS containing 0.1% Triton X-100 for 15 min at RT before further wash steps. Cells were blocked with 3% bovine serum albumin (BSA) in PBS-T for 1h at RT and then primary antibodies were incubated in the same blocking buffer for 1h at RT. Cells were washed three times and incubated with secondary antibodies in the blocking buffer (1:1000 dilution) for 1h at RT in dark. After three washes, coverslips were mounted onto glass microscope slides using ProLong Gold antifade reagent with the nuclear stain DAPI (Invitrogen). Before being visualized, the samples were stored in dark at 4°C. Confocal images were captured using a Leica SP5 Confocal microscope. All the images were acquired at 40x magnification. Representative images of the experiments are shown. Non-specific immunoreactivity was assessed by including a negative control where the primary antibodies were omitted (given that the images were negative they are not shown in the experiments).

Primary antibodies	Supplier
BRCA1 (I-20)	Santa Cruz Biotechnology
BRCA1 (D-20)	Santa Cruz Biotechnology
BRCA1 (MS110)	Millipore
gD (LP2)	A gift of Dr. Colin Crump
GFP (ab290)	Abcam
GFP (JL8)	A gift of Dr. Colin Crump

Secondary antibodies	Supplier
Alexa Fluor 488 goat anti-rabbit	Invitrogen
Alexa Fluor 488 goat anti-mouse	Invitrogen
Alexa Fluor 488 goat anti-mouse IgG2a	Invitrogen
Alexa Fluor 488 goat anti-mouse IgG1	Invitrogen
Alexa Fluor 568 donkey anti-mouse	Invitrogen
Alexa Fluor 568 goat anti-rabbit	Invitrogen
Alexa Fluor 568 goat anti-mouse IgG2a	Invitrogen
Alexa Fluor 633 goat anti-mouse	Invitrogen
Alexa Fluor 633 goat anti-mouse IgG1	Invitrogen

Table M2. Primary and secondary antibodies used for immunofluorescence analysis. The commercial source or the academic lab that provided the antibody are listed.

FACS fluorescence activated cell sorting

GFP positive U2OS stable cells were isolated by the Flow Cytometry Facility of the University of Cambridge.

Molecular cloning of IFI16

Polymerase Chain Reaction (PCR)

PCR was performed to amplify IFI16 incorporating the c-myc tag. PCR was performed in a final volume of 50 µl consisting of 5 µl 10x Buffer, 200 µM of each dNTP, 0.5 µM of each primer, 2,5 U Pwo polymerase (Roche), 2.5 mM MgCl2 and 10 ng of pCMV-SPORT6-IFI16B (NM_005531) (IRATp970A0119D, Source Bioscience) as template DNA. PCR conditions used for amplification of IFI16 were as follows: initial denaturation at 95°C for 5 min (1 cyle), denaturation at 95°C for 30 sec,

annealing at 55**°C for** 1 min, extension at 68°C for 6 min (25 cycles) and final extension at 68°C for 10 min. PCR product was run on a 1% agarose gel.

Primer	5'-3' sequence
IFI16 forward	CGG GAT CCG CCA CCA TGG GAA AAA AAT ACA AGA ACA TTG
IFI16 reverse c-myc	G CTG CGG CCG CTA CAG ATC TTC TTC AGA AAT AAG TTT TTG TTC GGT ACC GAA GAA AAA GTC TGG TGA AG

Table M3. Primers designed to amplify the target sequence.

Agarose gel electrophoresis

Gels were made using 100 ml TAE buffer, 1 g agarose and 3 µl ethidium bromide. Prior to electrophoresis, samples were mixed with DNA loading buffer and loaded onto the gel for electrophoresis at 100 V. Gels were visualized using UV light.

Gel purification of DNA fragments

DNA fragment of interest was gel purified using QIAquick® Gel Extraction kit (Qiagen) according to manufacturer's instructions.

Digestion and ligation of DNA fragments

DNA fragments were digested with the restriction enzymes BamHI and NotI (New England Biolabs) and cloned into the corresponding site of pHR-SIN cPPT-SGW vector (Demaison, Parsley et al. 2002) a gift of Dr. Paul Lehner (Cambridge Institute for Medical Research, Department of Medicine, University of Cambridge). Ligation of DNA fragments was carried out by incubation of the linearised and pre-digested vector with the DNA insert at a 1:4 ratio, 5 U of T4 DNA ligase (New England Biolabs) and T4 DNA ligase buffer in a final volume of 10µl for 2 h at RT.

Transformation

Competent bacteria (*Escherichia coli* strain XL1 blue) were incubated with the plasmid of interest for 30 min on ice. Bacteria were heat-shocked at 37°C for 3 min and transferred to ice for further 2 min. Following the addition of SOB broth, the bacteria were incubated at 37°C on a shaker for 1h and spread onto agar plates containing ampicillin antibiotic. After overnight incubation at 37°C single colonies were picked and placed into LB culture medium containing 100µg/ml ampicillin for 8 h at 37°C on a shaker.

Plasmid purification

Plasmids were isolated using QUIAprep® Spin Miniprep kit (Qiagen) according to manufacturer's instructions. The construct was verified by digesting and sequencing.

DNA sequencing

Sequencing was carried out by Parque Científico de Madrid and the analysis of the sequence was performed using DNASTAR Lasergene 8 software.

Transfections

Plasmids were transfected with jetPEI™ (Polyplus transfection) in OptiMEM® medium (GIBCO) following manufacturer's instructions.

Plasmids	Supplier
pHR-SIN-IFI16	Described in this thesis
pWZL hygro (empty vector)	Dr. M Serrano
pWZL hygro hp53 wt	Dr. M Serrano
pWZL hygro hp53 175H	Dr. M Serrano (Serrano, Lin et al. 1997)
GFP-UL37	Dr. Colin Crump
GFP-UL37_N-terminus	Dr. Colin Crump
GFP-UL37_ΔN-terminus	Dr. Colin Crump

Table M4. Plasmids used in this study.

Results

1. *Study of IFI16 protein in HSV-1 infection:*

As described in the introduction, host cells have developed mechanisms to detect and restrict viral infections, but in response, viruses have also evolved a large variety of strategies to evade these immune processes. Several viruses including herpesviruses use viral factors that are similar to E3 ubiquitin ligases to inactivate the host cell defenses. The HSV-1 immediate-early protein ICP0 is an example of these viral factors, and previous studies have shown that, by acting as an E3 ubiquitin ligase, ICP0 promotes the degradation or dissociation of several cellular proteins to counteract the antiviral responses (reviewed in (Boutell and Everett 2013; Lanfranca, Mostafa et al. 2014)).

Among the diverse range of proteins targeted by ICP0 is IFI16, which acts as a nuclear DNA sensor during HSV-1 infection (Orzalli, DeLuca et al. 2012; Li, Diner et al. 2012a; Johnson, Chikoti et al. 2013) to induce the expression of type I IFNs (Unterholzner, Keating et al. 2010; Orzalli, DeLuca et al. 2012) and the maturation of IL-1β (Johnson, Chikoti et al. 2013). In addition to its roles in IFN-β induction and activation of the inflammation response, IFI16 is also involved in the silencing of the HSV-1 genome, promoting the assembly of heterochromatin on viral DNA (Orzalli, Conwell et al. 2013) and reducing the association of important transcription factors with promoters of several HSV-1 genes (Johnson, Bottero et al. 2014), therefore limiting the viral replication and viral gene expression.

1.1. Analysis of IFI16 levels after HSV-1 infection: Effect of ICP0 expression on IFI16 stability.

Orzalli *et al*. were the first to report a marked reduction in the levels of IFI16 in human foreskin fibroblasts (HFF) infected with HSV-1 and to demonstrate that ICP0 promoted the IFI16 degradation in a proteasome- and RING finger domain-dependent mechanism (Orzalli, DeLuca et al. 2012).

To confirm their findings regarding the ICP0-induced decrease in IFI16 levels, human telomerase reverse transcriptase (hTERT) immortalized HFF cells were mock infected (uninfected) or infected at multiplicity of infection (m.o.i.) 10 with an ICP0 deletion mutant HSV-1 (*dl*1403), the control revertant virus (*dl*1403R) and a mutant HSV-1 deficient in all the immediate early (IE) genes except ICP0 (dh1f) (Table R1). The *dl*1403 mutant expresses a truncated form of ICP0 that has been shown to be inactive. The virus used as wild type (wt) is the revertant of the *dl*1403 mutant in which the ICP0 deleted sequences have been restored (Stow and Stow 1986). Whole cell lysates were prepared 24 hours post infection (hpi) and western blot analysis performed with antibodies to ICP4 as a control, ICP0, IFI16 and β-actin (Fig. R1).

HSV-1 virus	Mutation
*dl*1403	ICP0-
*dl*1403R	dl1403 revertant
dh1a	gH-
dh1b	gH-,ICP4-
dh1c	gH-,ICP4-,ICP22/47-
dh1f	gH-,ICP4-,ICP22/47-,ICP27-

Table R1. HSV-1 viruses used in this study.

Like Orzalli *et al*, the levels of IFI16 protein in HFF cells were observed to decrease dramatically after infection with the revertant HSV-1 (*dl*1403R) and the mutant deficient in all the IE genes except ICP0 (dh1f), but not after the infection with the ICP0 deletion mutant virus (*dl*1403). Staining for β-actin confirmed similar loading of both mock and infected cell lysates (Fig. R1).

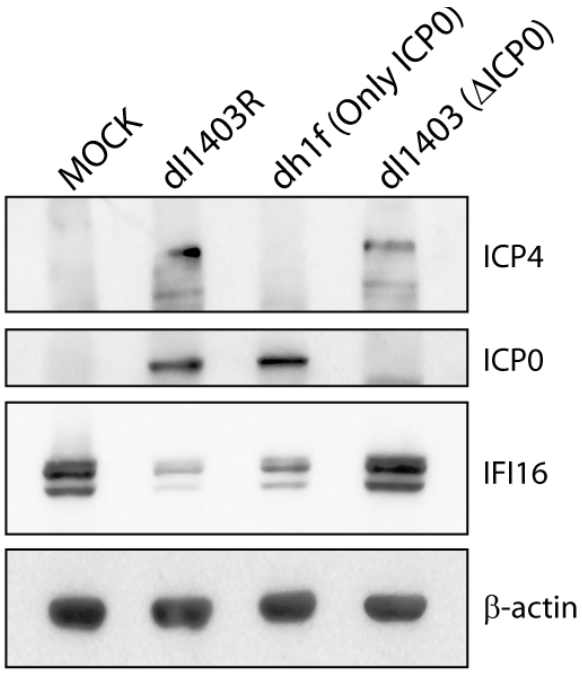


Figure R1. HSV-1 infection induces a decrease in IFI16 levels in an ICP0-dependent manner. HFFs were uninfected or infected with a control revertant HSV-1 (*dl*1403R), a mutant HSV-1 that expresses ICP0 but not additional IE genes (dh1f) or an ICP0 deletion mutant virus (*dl*1403) at 10 pfu/cell. Total cell lysates were prepared at 24 hpi and 50 µg were analyzed by western blot with antibodies to ICP4 as a control, ICP0, IFI16 and β-actin.

These data confirm the findings of Orzalli *et al.* regarding the reduction in the levels of IFI16 after HSV-1 infection in HFFs when ICP0 is expressed.

Given that the effect of HSV-1 infection on IFI16 levels had only been studied in HFFs (Orzalli, DeLuca et al. 2012), it was interesting to test whether this decrease in IFI16 levels was cell type-dependent. To analyze if this result was consistent in other human cell lines permissive for HSV-1 infection, human osteosarcoma (U2OS) cells were infected with the mutant virus defective in ICP0 (*dl*1403), the revertant virus (*dl*1403R) and a mutant virus defective in the glycoprotein H (dh1a) as a control (Table R1) (all infections were done using a m.o.i. of 10). Since the dh1 mutant virus series derived from gH-null HSV-1 (dh1b, dh1c and dh1f), used in the following experiments, cannot express ICP4, an antibody for VP16 (an HSV-1 tegument protein) was used as a control of infection, unless otherwise indicated. Lysates were prepared at 24 hpi and western blot analysis performed (Fig. R2).
The result obtained in U2OS cells was consistent with the previous data from HFF infection studies since IFI16 levels were only diminished after infection with those viruses that express ICP0, which are the revertant virus *dl*1403R and the mutant dh1a (Fig. R2). However, the HSV-1 induced decrease in IFI16 levels was less marked than in HFF cells. Given that ICP0 is not required for efficient HSV-1 replication in U2OS (Yao and Schaffer 1995), it is perhaps possible that ICP0 is not as functionally important during infection of U2OS cells and that it cannot counteract the effect of IFI16 with the same efficiency as in HFF cells.

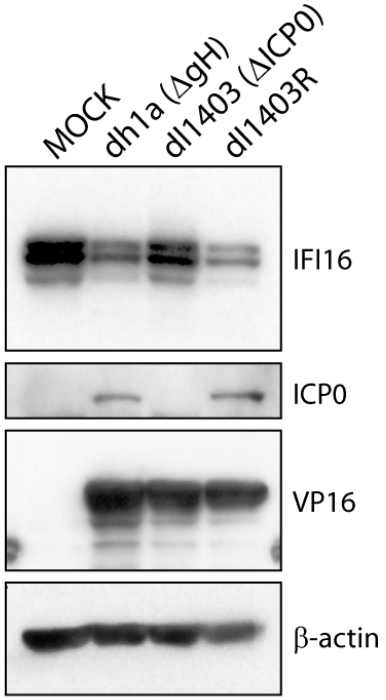


Figure R2. HSV-1 infection induces a decrease in IFI16 levels in an ICP0-dependent manner. U2OS cells were uninfected or infected with a mutant deficient in gH (dh1a), the mutant deficient in ICP0 (*dl*1403) or the control revertant virus (*dl*1403R) at 10 pfu/cell. Total cell lysates were prepared at 24 hpi and 50 µg were analyzed by western blot with antibodies to VP16 as a control of infection, IFI16, ICP0 and β-actin.

During the course of this work, another paper was published reporting that HSV-1 infection in HFFs induced IFI16 degradation in an ICP0- and proteasome-dependent mechanism (Johnson, Chikoti et al. 2013). This study confirmed the data of Orzalli et al. and our results showing that HSV-1 infection of HFFs produced a decrease in IFI16 levels in an ICP0-dependent manner.

However a third study, from Roger Everett's group, presented data that are in apparent contrast, since they indicated that ICP0 is neither sufficient nor necessary for the degradation of IFI16 during the course of HSV-1 infection (Cuchet-Lourenco, Anderson et al. 2013). They suggested that IFI16 degradation was dependent on viral progression independently of ICP0 expression, but they did not discard an indirect effect of ICP0 in IFI16 degradation.

To resolve this discrepancy, and to clarify whether the loss of IFI16 expression was an ICP0-independent consequence of the progression of viral infection, HFF and U2OS cells were infected with mutants of HSV-1 deficient for the expression of a range of IE proteins, but which express ICP0 (dh1b, dh1c and dh1f) (Table R1).

The mutants dh1b, dh1c and dh1f express ICP0, but are unable to complete a normal cycle of viral replication due to the absence of the major transcriptional activator ICP4, thus analysis of IFI16 levels in cells infected with these viruses allows discrimination between the effects of ICP0 expression and those of the progression of viral infection. In these experiments the levels of IFI16 expression were dramatically lower in the HFFs infected with all the mutants that express ICP0 (dh1a, dh1b, dh1c and dh1f) than in those cells infected with the mutant in ICP0 (*dl*1403) (Fig. R3-A). Thus these data confirm that ICP0 is a key factor that governs IFI16 stability in HFFs.

IFI16 levels were also decreased in U2OS infected with the mutants that express ICP0 (dh1a, dh1b, dh1c and dh1f) (Fig. R3-B), but consistent with our previous data, the effect was less marked than in HFF cells. However, in this experiment the loss of IFI16 during the control revertant (*dl*1403R) infection of U2OS cells was more similar to that in HFF cells (compare Figs. 3A and 3B) than in previous experiments (compare Figs. R1 and R2). Cuchet-Lourenço *et al*. also observed less IFI16 degradation in U2OS infected with the wt virus than in other cells examined, including HFs (human diploid fibroblasts) and HepaRG cells (a human hepatocellular carcinoma cell line). Although in our experiments more IFI16 degradation was observed in the U2OS cells infected with the control virus than that seen by Cuchet-Lourenço and colleagues. This may be explained because here the expression of IFI16 was analysed after 24 hpi whereas Cuchet-Lourenço *et al.* assayed for IFI16 12 hpi. Moreover, they observed only a slight decrease in the amounts of IFI16 after the infection with the virus deficient in ICP0 in U2OS cells whereas in HepaRG cells they noted that the rate of IFI16 loss was equivalent in the infection with the wt and with the mutant in ICP0 when progression of infection was equivalent in both infections. From these studies they concluded that the efficiency of IFI16 degradation in response to HSV-1 infection is different depending on the cell type and therefore the cellular factors that govern

the IFI16 degradation could be defective in U2OS cells. As explained before, another possibility is that the lesser degradation of IFI16 in the U2OS cells infected with the wt virus compared to other cell lines is because the pathways targeted by ICP0 are defective/inefficient in U2OS cells (Yao and Schaffer 1995; Hancock, Cliffe et al. 2010). This hypothesis is consistent with the observation that the U2OS cells infected with the mutants defective in the rest of the IE genes but express ICP0 (dh1b, dh1c and dh1f) showed less IFI16 loss than HFF cells. Although ICP0 may play an important role since the U2OS cells infected with the ICP0 mutant virus which expresses the rest of the IE genes (*dl*1403) showed less IFI16 degradation than the mutant that only expresses ICP0 (dh1f).

To test the hypothesis of Cuchet-Lourenço *et al*. regarding the different IFI16 degradation according to the cell type, the stability of IFI16 was analysed in another cell line, the Vero cell line, derived from the kidney of an African green monkey, which is unable to synthesize interferon (Desmyter, Melnick et al. 1968; Emeny and Morgan 1979) and hence highly permissive to viral infection (Rhim, Schell et al. 1969).

In Vero cells the levels of IFI16 maintained the same pattern (Fig. R3-C) as seen in U2OS cells when infected with the different HSV-1 viruses, although the loss of IFI16 was lower than in U2OS cells (Figs. R2 and R3-B). However it is important to note that Vero cells differ from the previous cell lines analysed in that Vero cells only expressed one of the three IFI16 isoforms (Fig. 3C).

These results showed that the expression of IFI16 displayed a similar trend in HFF, U2OS and Vero cells after HSV-1 infection, thus reconfirming the necessity of ICP0 for IFI16 degradation. Furthermore, these data suggest that the rate of IFI16 degradation does not correlate with an efficient viral progression, because although the mutants dh1b, dh1c and dh1f are unable to complete a normal cycle of viral replication, the cells infected with these mutant viruses showed a decrease in IFI16 levels. However, the degree of IFI16 reduction in cells infected with the mutant dh1f did not reach that observed in control virus infection, indicating that IFI16 degradation depends on other factors in addition to ICP0. Nevertheless, a control for ICP0 expression and viral progression of infection would have been required to allow more confident interpretation of the data.

Therefore, it was necessary to reexamine whether ICP0 was the main factor that orchestrates the loss of IFI16 or whether it was an indirect effect, as according to Cuchet-Lourenço *et al*. the degradation of IFI16 correlates with infection progression. And given that the mutant viruses that lack functional ICP0 show a replication defect (Everett, Boutell et al. 2004), the absence of IFI16 degradation in cells infected with the ICP0-null mutant virus could be explained due to the delay in the infection progression rather than due to the absence of ICP0 *per se*.

Therefore, to further explore the hypothesis of Cuchet-Lourenço *et al*. and discard the possibility

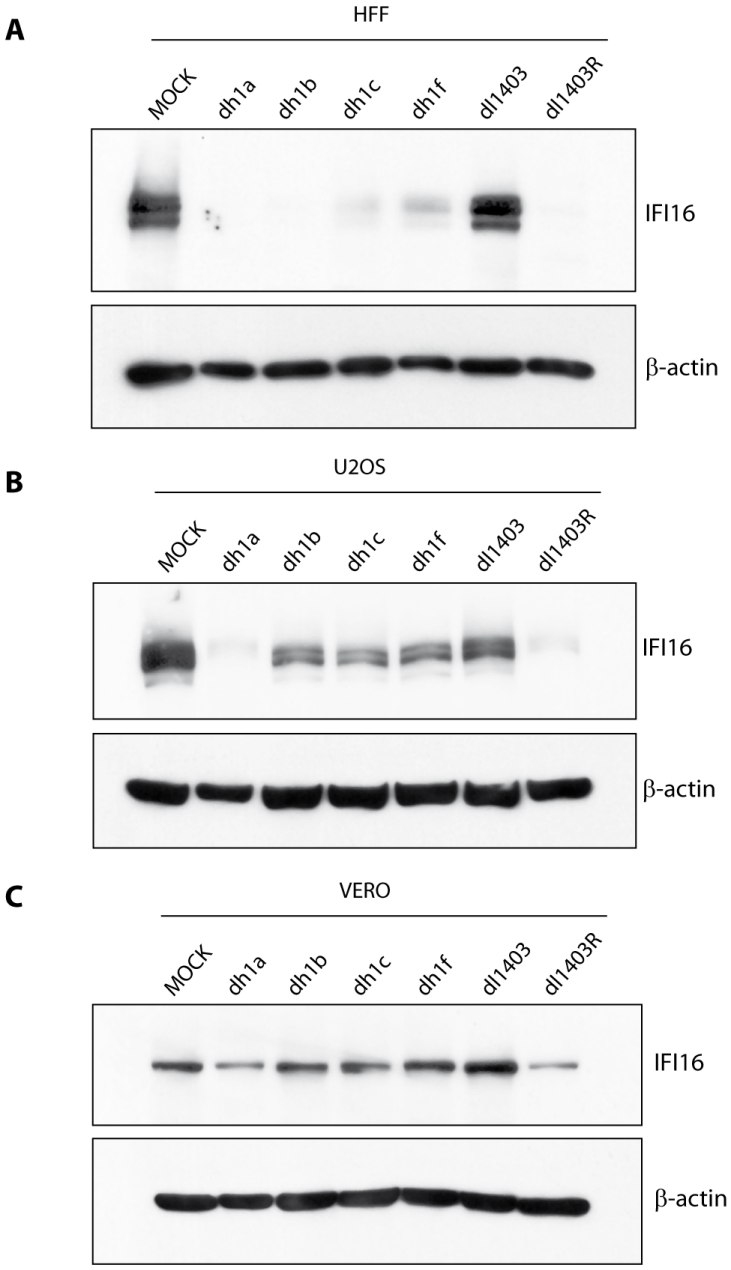


Figure R3. ICP0 is necessary for the decrease in IFI16 levels during HSV-1 infection. (A) HFF, **(B)** U2OS and **(C)** Vero cells were infected with different mutants in gH (dh1a), IE genes (dh1b, dh1c, dh1f and dl1403) and the ICP0 rescued virus (dl1403R) at 10 pfu/cell. Total cell lysates were prepared at 24 hpi and 20 µg were analyzed by western blot with antibodies to IFI16 and β-actin.

that the observed absence of IFI16 loss in cells infected with the ICP0-null mutant was due to a delay in the viral progression, the IFI16 levels were analysed in cells infected with the mutant in ICP0 (*dl*1403) at higher m.o.i. than that of the control virus (*dl*1403R). This experiment was performed firstly with U2OS because if ICP0 is not required for efficient HSV-1 replication in U2OS cells (Yao and Schaffer 1995), the infections with the control virus and the ICP0-null mutant virus would be expected to progress equally efficiently. However Cuchet- Lourenço *et al.* found that the viral gene expression during ICP0-null mutant infection of U2OS cells was a bit delayed; therefore the experiment was performed using the control virus at an m.o.i of 20 and the mutant in ICP0 at an m.o.i. of 45. The other mutants were infected using an m.o.i. of 20 (Fig. R4). As expected, the levels of IFI16 were not decreased during ICP0-null mutant virus (*dl*1403) infection, although it seems, as judged by the different rates of gD expression, that this infection did not progress equally efficiently as the control

(*dl*1403R) infection (Fig. R4). gD glycoprotein is incorporated into the viral particle and part of the gD detected represents glycoprotein persisting from the input virus used for the infection. Given that gD staining was not the most appropriate control for viral progression, we decided to use another viral protein synthesized *de novo* as a control of viral progression. The expression of UL42, a protein that is early expressed during HSV-1 infection, was used to compare the rates of viral progression (Fig. R4). As can be observed from the UL42 expression levels, the infection with the mutant in ICP0 did not progress as efficiently as the control infection, even using 2.25 more pfu/cell. On the other hand, another observation that supports our hypothesis is that the cells infected with the mutants dh1b, dh1c and dh1f present a defective progression of infection (observe UL42 staining) due to the absence of ICP4, however, they still did show IFI16 degradation (Figs. R3 and R4).

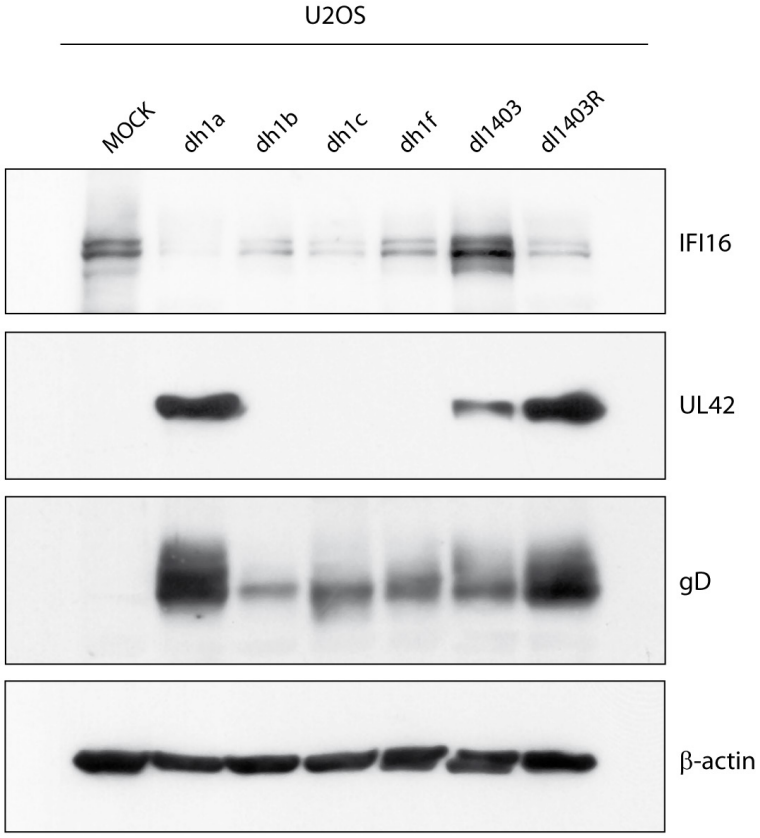


Figure R4. **ICP0 is necessary for the decrease in IFI16 levels during HSV-1 infection**. U2OS were infected with the mutant *dl*1403 at 45 pfu/cell, and with the revertant virus (*dl*1403R) and the mutant virus dh1a, dh1b, dh1c and dh1f at 20 pfu/cell. Total cell lysates were prepared at 24 hpi and 20 µg were analyzed by western blot with antibodies to IFI16, UL42, gD and β-actin.

To further explore the previous result and ensure that the infection with the ICP0-null mutant virus progressed equally efficiently, U2OS cells were infected with a greater difference of m.o.i using the mutant *dl*1403 virus at 20 pfu/cell and the revertant virus *dl*1403R at 4 pfu/cell (Fig. R5).

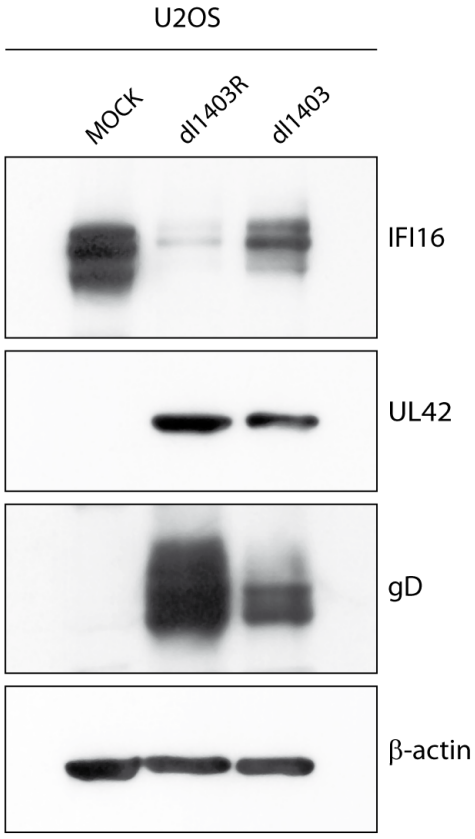


Figure R5. **IFI16 levels are slightly decreased in U2OS cells infected with the ICP0 mutant when the viral infection progress efficiently.** U2OS were infected with the mutant *dl*1403 at 20 pfu/cell and with the corresponding rescued virus *dl*1403R at 4 pfu/cell. Total cell lysates were prepared at 24 hpi and 20 µg were analyzed by western blot with antibodies to IFI16, UL42, gD and β-actin.

Even in this condition, the result obtained in U2OS was consistent with our previous data since IFI16 levels were once again dramatically diminished after infection with the control virus comparing with the ICP0 mutant virus infection (Fig. R5). However, despite the mutant virus being used at higher multiplicity, this infection still progressed slightly less efficiently than the control infection as judged by the UL42 levels. The levels of gD detected in cells infected with the mutant might be expected to be similar than those observed after control infection since the mutant virus was used at higher multiplicity and part of the gD detected represents glycoprotein persisting from the input virus used for the infection. Moreover, the mutant infection progressed similarly to control infection. However, the levels of gD after mutant infection were much lower than those from control infection. This may indicate that the differences in the progression rate were more pronounced at late stages of viral expression.

To extend this result, the levels of IFI16 were examined in HFFs infected with the control virus at m.o.i of 10 or 20 and with the mutant in ICP0 at much higher multiplicity, m.o.i of 200 or 250, because HFFs are less permissive for the mutant infection (Fig. R6). IFI16 levels were still

slightly diminished in the cells infected with the ICP0 mutant virus (*dl*1403) even though the infection progressed with almost comparable efficiency to the control infection in the HFF cells of the left panel, as shown by the UL42 expression levels. However, the mutant virus infection still progressed less efficiently than the control virus infection.

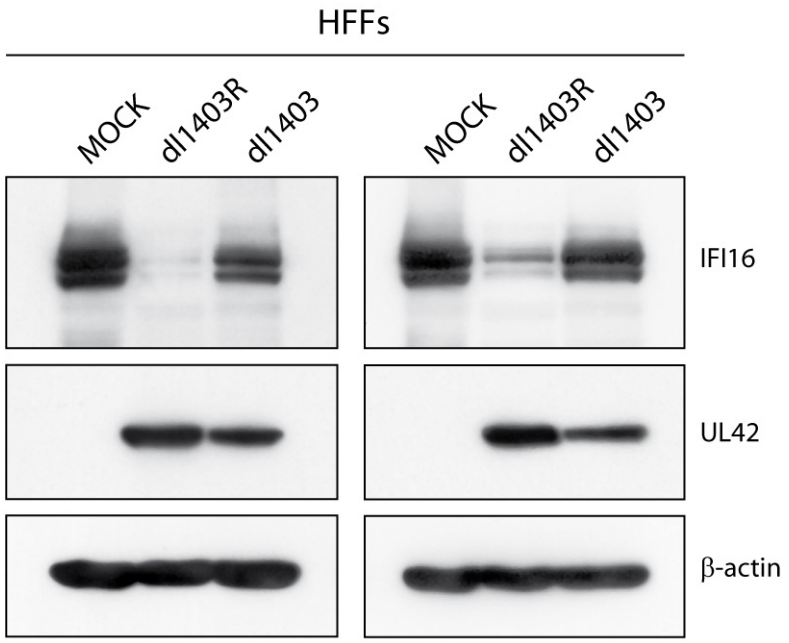


Figure R6. **IFI16 levels are slightly or not decreased in HFF cells infected with the ICP0 mutant virus when the viral infection progress efficiently.** HFF were infected with the mutant *dl*1403 at 200 pfu/cell (left panel) or 250 pfu/cell (right panel) and with the corresponding rescued virus *dl*1403R at 10 pfu/cell (left panel) or 20 pfu/cell (right panel). Total cell lysates were prepared at 24 hpi and 30 µg were analyzed by western blot with antibodies to IFI16, UL42 and β-actin.

Altogether, these data confirm that ICP0 plays a role in IFI16 degradation. The involvement of ICP0 was confirmed from results obtained by study of three independent cell lines: two human cell lines, normal (HFFs) and tumoral (U2OS), and a monkey cell line (Vero). Despite their different origins, all of them displayed the same pattern of IFI16 destabilization in an ICP0-dependent manner.

During the writing of this work, another study has observed that ICP0 is necessary but not sufficient for IFI16 degradation (Diner, Lum et al. 2015b), suggesting another viral or cellular mechanism, different from other ICP0 targets, that contributes to IFI16 degradation.

In view of the findings from other groups and our own results, it is tempting to conclude that ICP0 is essential for an efficient IFI16 degradation, although the degree of IFI16 loss varies between different cell lines, implying that other factors still unknown are involved in IFI16 degradation.

1.2. Analysis of the stability of IFI16-interacting proteins after HSV-1 infection:

As explained in the introduction, in addition to its role as a sensor for pathogen DNA in the innate immune response, IFI16 has also been implicated in the DNA damage response (DDR) due to its physical interaction with the tumor suppressor proteins p53 and BRCA1 (Johnstone, Wei et al. 2000; Aglipay, Lee et al. 2003; Song, Alimirah et al. 2008; Liao, Lam et al. 2011).
The HIN-A domain of IFI16 binds the C-terminus of p53, likely preventing nonspecific p53-DNA interactions, whereas the HIN-B domain of IFI16 recognizes the core domain of p53 stabilizing p53 binding to DNA (Liao, Lam et al. 2011). On the other hand, the PYRIN domain of IFI16 interacts with BRCA1 promoting p53-dependent apoptosis in presence of cellular damage or oncogenic stimuli. Indeed, IFI16 is a component of the DNA repair multi-protein complex known as BASC (BRCA1-associated genome surveillance complex) *(Aglipay, Lee et al. 2003)*. In turn, both p53 and BRCA1 are proteins known to interact with the HSV-1 genome (Wilcock and Lane 1991; Maul, Jensen et al. 1998; Lilley, Chaurushiya et al. 2011; Hsieh, Kuta et al. 2014).

The tumor suppressor p53 is critical for antiviral defense by inducing apoptosis and enhancing type I IFN responses (reviewed in (Rivas, Aaronson et al. 2010)). In the context of HSV-1 infection, an early study showed that p53 is recruited to nuclear HSV-1 DNA replication compartments (Wilcock and Lane 1991) and more recently two p53 responsive elements (p53RE) have been identified in the HSV-1 genome, where p53 binds both *in vitro* and *in vivo* suggesting a mechanism of viral gene regulation by p53 (Hsieh, Kuta et al. 2014).

BRCA1 plays a central role in DNA repair controlling the cellular responses to DNA damage (Scully, Chen et al. 1997a; Scully, Chen et al. 1997b). Like other DNA repair proteins, the incoming HSV-1 genomes affect BRCA1 protein since BRCA1 accumulations are dispersed from nuclear domains after wild type HSV-1 infection (Maul, Jensen et al. 1998), leading to a redistribution of BRCA1 in accumulations very close to incoming viral genomes, but this early response to HSV-1 infection is inhibited by ICP0 (Lilley, Chaurushiya et al. 2011).

Whether IFI16 recognizes foreign DNA together with other proteins such as p53 and BRCA1 is unknown. Knowing that there are direct interactions between IFI16 protein with BRCA1 and p53, the expression levels of these proteins were analysed after HSV-1 infection. Our hypothesis was that as IFI16 is degraded during HSV-1 infection, the levels of these proteins could also decrease after HSV-1 infection if they are interacting directly with IFI16 in the same complex to bind HSV-1 DNA. However, the degradation of p53 and/or BRCA1 would not necessarily imply that they interact with IFI16 to bind the viral DNA, and vice versa the absence of p53 and/or BRCA1 degradation would not necessarily mean that these proteins do not participate with IFI16 in the early recognition of HSV-1 genome.

1.2.1. Study of p53 stability after HSV-1 infection:

The tumor suppressor p53 is a short-lived protein whose levels are maintained low in normal cells mainly by the action of Mdm2 (double minute 2 protein), a cellular ubiquitin ligase (Haupt, Maya et al. 1997). However, after stress stimuli p53 is stabilized and activated by post-translational modifications that affect its interaction with Mdm2 (reviewed in (Lavin and Gueven 2006)). Therefore the stabilization of p53 is crucial to initiate an efficient response to cellular stress.

Viral infections represent an important cellular stress and, as mentioned before, p53 is critical for the antiviral defense. The transcription of the p53 gene is induced by type I IFNs due to the presence of ISREs (IFN-stimulated response elements) in its promoter (Takaoka, Hayakawa et al. 2003). Moreover, p53 is activated indirectly by type I IFNs to induce apoptosis in the infected cells (Takaoka, Hayakawa et al. 2003; Townsend, Scarabelli et al. 2004; Ding, Lee et al. 2006; Pampin, Simonin et al. 2006) and in turn p53 enhances type I IFN signaling and production because several IFN-inducible genes (IRF9, IRF5, ISG15 and TLR3) are direct transcriptional targets of p53 (Hummer, Li et al. 2001; Mori, Anazawa et al. 2002; Munoz-Fontela, Macip et al. 2008; Taura, Eguma et al. 2008). To overcome the proapoptotic and antiviral activities of p53, some viruses have evolved mechanisms to counteract the p53 responses, for example leading to p53 degradation (Scheffner, Werness et al. 1990; Moore, Horikoshi et al. 1996; Querido, Marcellus et al. 1997; Steegenga, Riteco et al. 1998; Querido, Blanchette et al. 2001; Pampin, Simonin et al. 2006).

During HSV-1 infection, previous studies have demonstrated that although ICP0 promotes the ubiquitination of p53 (Boutell and Everett 2003), p53 levels were actually stabilized in HSV-1 infection of HFF-2 cells, a nontransformed human primary cell line derived from foetal foreskin fibroblasts, and this stabilization occurred independently of ICP0 (Boutell and Everett 2004; Boutell, Canning et al. 2005).

However the stabilization of p53 by HSV-1 infection was cell type dependent since the wt p53 expressed by U2OS cells was not stabilized during HSV-1 infection, in fact the total levels of p53 were reduced independently of the presence or absence of ICP0 (Boutell and Everett 2004).

To extend these previous results, the p53 levels were examined after HSV-1 infection in the HFF and U2OS cell lines used in our previous experiments (Figs. R1 and R2).

In HFFs the p53 levels were stabilized after infection with the control HSV-1 (*dl*1403R) (Fig. R7), in agreement with the previous results from Boutell and colleagues (Boutell and Everett 2004; Boutell, Canning et al. 2005), while infection with *dl*1403, at elevated m.o.i. to overcome the requirement for ICP0-mediated transactivation, resulted in levels of p53 that were only somewhat lower than those observed in control virus infection (Fig. R7). Infection with a mutant virus that expresses ICP0 but no additional IE gene products (dh1f) showed no increase in p53 stabilization compared to mock cells (Fig. R7). These results suggest that p53 stabilization in HFF cells does not depend exclusively on ICP0, although its expression helps p53 stabilization. As Boutell *et al.* proposed it is possible that p53 stabilization requires the ability of ICP0 to induce viral gene expression (Boutell

and Everett 2004).

The data obtained in U2OS cells were also consistent with previously published results from Boutell and Everett (Boutell and Everett 2004) since p53 levels were not stabilized after HSV-1 infection independently of ICP0 expression (Fig. R8).

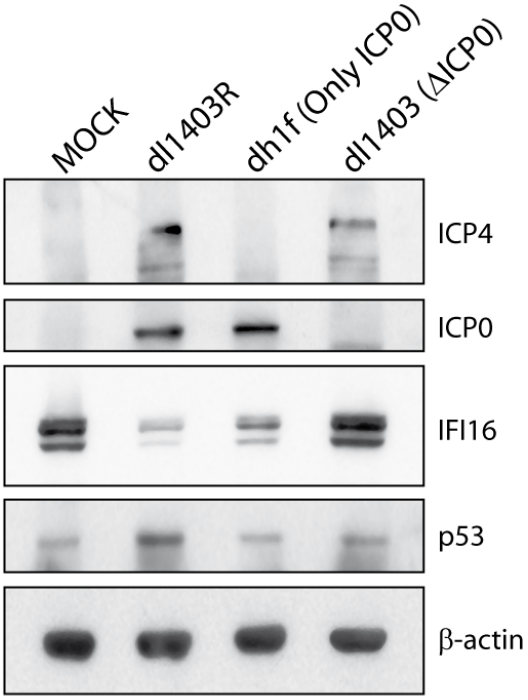


Figure R7. HSV-1 infection of HFF cells induces an increase in p53 levels in an ICP0-independent manner. HFFs were uninfected or infected with a control revertant HSV-1 (*dl*1403R), a mutant HSV-1 that expresses ICP0 but not additional IE genes (dh1f) or an ICP0 deletion mutant virus (*dl*1403) at 10 pfu/cell. Total cell lysates were prepared at 24 hpi and 50 µg were analyzed by western blot with antibodies to ICP4, ICP0 IFI16, p53 and β-actin.

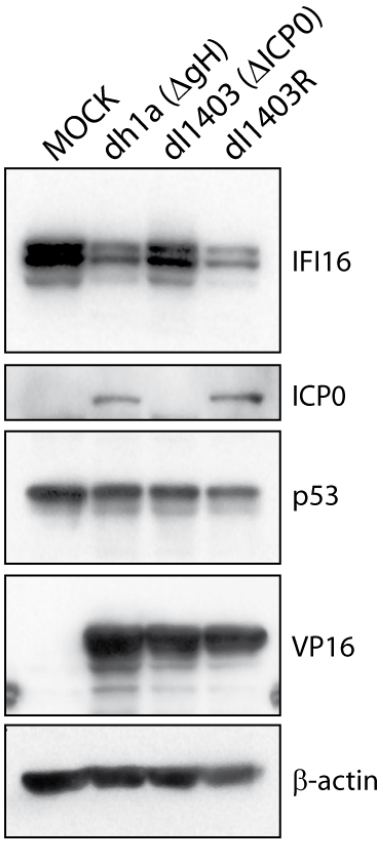


Figure R8. HSV-1 infection of U2OS cells induces a slight decrease in p53 levels in an ICP0-independent manner. U2OS cells were uninfected or infected with a mutant deficient in gH (dh1a), the mutant deficient in ICP0 (*dl*1403) or the control revertant virus (*dl*1403R) at 10 pfu/cell. Total cell lysates were prepared at 24 hpi and 50 µg were analyzed by western blot with antibodies to IFI16, ICP0, p53, VP16 and β-actin.

In conclusion, these results support previous published findings and confirmed that HSV-1 infection does not reduce p53 stability in an ICP0-dependent manner, arguing against the idea that HSV-1 ICP0 targets IFI16 complexed with p53. However, this result does not necessarily imply that p53 does not interact with IFI16 to bind the HSV-1 DNA.

1.2.2. Study of BRCA1 stability after HSV-1 infection:

As stated in the introduction, infection by many viruses induces the activation of the DNA damage signaling pathways through the same cellular cascades that are activated by genomic DNA damage. And in turn, viruses have developed complex mechanisms to inactive or activate these pathways in order to facilitate viral replication (reviewed in (Turnell and Grand 2012)).

HSV-1 has a complex relationship with the DNA damage and repair machinery. During HSV-1 infection, DNA repair proteins are activated and accumulated at sites of viral genomes (Lilley, Chaurushiya et al. 2011). Some of them are beneficial for HSV-1 replication (Taylor and Knipe 2004; Lilley, Carson et al. 2005; Mohni, Livingston et al. 2010; Placek and Berger 2010) while others are detrimental to HSV-1 replication (Parkinson, Lees-Miller et al. 1999; Taylor and Knipe 2004; Lilley, Chaurushiya et al. 2011). However, the antiviral activity of the proteins deleterious for the viral infection is overcome in part through the E3 ubiquitin ligase activity of ICP0. Specifically ICP0 ubiquitinates and directs the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (Lees-Miller, Long et al. 1996; Parkinson, Lees-Miller et al. 1999) and the DNA damage E3 ubiquitin ligases RNF8 and RNF168 (Lilley, Chaurushiya et al. 2010).

As described in the introduction, DNA-PK plays a central role in DNA double-strand break repair via non-homologous end-joining (NHEJ) pathway and in V(D)J recombination. In addition it plays newly emerging roles in mitosis, transcription, maintenance of telomeres and in viral infections (reviewed in (Jette and Lees-Miller 2015)). In fact, it has been shown that DNA-PK acts as a DNA sensor that induces the production of type I IFN, cytokines and chemokines in an IRF-3-dependent manner during infection with DNA viruses, such as HSV-1 (Ferguson, Mansur et al. 2012). RNF8 and RNF168 are key mediators in the ATM-dependent signaling pathway. They are cellular RING finger E3 ubiquitin ligases that play a central role in the DNA double-strand break response by poly-ubiquitinating histones, a signal that promotes the accumulation of the downstream effectors such as 53BP1 and BRCA1 to the damaged chromatin (Huen, Grant et al. 2007; Kolas, Chapman et al. 2007; Mailand, Bekker-Jensen et al. 2007; Wang and Elledge 2007; Doil, Mailand et al. 2009). Similarly, RNF8 and RNF168 coordinate the recruitment of DNA repair factors to viral genomes during HSV-1 infection; therefore identifying both proteins as components of the host antiviral defense against HSV-1 (Lilley, Chaurushiya et al. 2011).

The tumor suppressor BRCA1 is a 220-KDa protein that plays roles in several pathways that

maintain genomic stability, including protein ubiquitination (reviewed in (Wu, Koike et al. 2008)), chromatin remodeling (Bochar, Wang et al. 2000), transcriptional regulation (reviewed in (Rosen, Fan et al. 2006)), apoptosis (Shao, Chai et al. 1996; Harkin, Bean et al. 1999) and the DNA-damage response pathways through DNA damage-induced cell cycle checkpoint activation (Xu, Kim et al. 2001; Yarden, Pardo-Reoyo et al. 2002) and DNA damage repair, particularly dsDNA breaks, via NHEJ (Zhong, Chen et al. 2002a; Zhong, Boyer et al. 2002b; Bau, Fu et al. 2004; Wang, Chou et al. 2006; Zhuang, Zhang et al. 2006) and homologous recombination (HR) (Moynahan, Chiu et al. 1999; Stark, Pierce et al. 2004). Like other DNA repair proteins, BRCA1 relocates to DNA damage sites forming nuclear foci (Scully, Chen et al. 1997b). As mentioned previously, what is known about BRCA1 during HSV-1 infection is that BRCA1 accumulations are dispersed from its nuclear domains in the initial phases of HSV-1 infection (Maul, Jensen et al. 1998) and redistributed proximal to incoming viral genomes, but this early response to HSV-1 infection is inhibited by ICP0 (Lilley, Chaurushiya et al. 2011).

Given that ICP0 induces the degradation of some proteins involved in DNA damage responses including the DNA sensor IFI16, which is known to interact with BRCA1 in the BASC complex, we wondered if ICP0 could also affect BRCA1 stability during HSV-1 infection.

To test the effect of ICP0 on BRCA1 levels, HFF and U2OS cells were infected with either the ICP0-null HSV-1 (*dl*1403) or the control revertant HSV-1 (*dl*1403R) at m.o.i. of 20. Lysates prepared from these cells 24 hpi were analysed by western blot using an antibody against BRCA1 (Santa Cruz I-20) (Fig. R9).

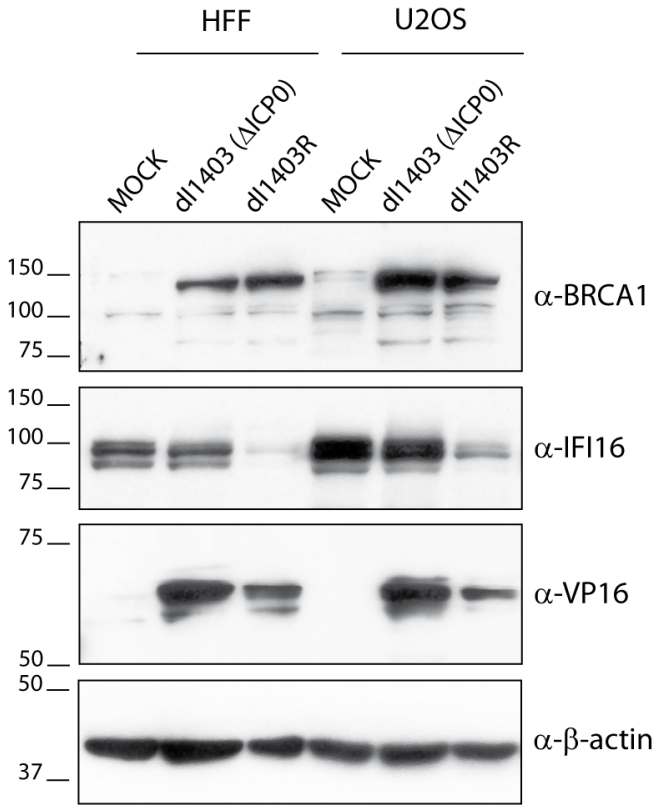


Figure R9. HSV-1 infection induces a marked increase in the levels of a 120-KDa protein reactive with an antibody specific for BRCA1 in an ICP0-independent manner in HFF and U2OS cells. HFF and U2OS cells were infected with the mutant deficient in ICP0 (*dl*1403) or the revertant virus (*dl*1403R) at 20 pfu/cell. Total cell lysates were prepared at 24 hpi and 25 µg were analyzed by western blot with antibodies to BRCA1 (I-20), IFI16, VP16 and β-actin.

Unlike IFI16 levels, which are decreased after infection with the control revertant virus but are maintained after infection with the ICP0 deletion mutant virus, HSV-1 infection with both the revertant and the mutant virus provoked a marked increase of an approximately 120-KDa protein reactive with the antibody for BRCA1 (Fig. R9), hereafter referred to as the 120 kDa protein.

Given that canonical BRCA1 protein has an apparent molecular weight on SDS-PAGE of around 220-230 KDa, this 120-KDa protein could be a potential variant of BRCA1, a product of BRCA1 cleavage induced by HSV-1 or another protein. In any case, it can be concluded from these data that ICP0 is not involved in the up-regulation of this protein. However, it is important to note that this conclusive data relating to the ability of HSV-1 to regulate BRCA1 expression levels could not be validated since detection of a protein at the previously reported molecular weight was not observed in our western blot analysis.

As described in the introduction, HSV-1 promotes a partial activation of the ATM-dependent signaling pathways, since infection induces the activation of ATM and downstream targets including Nbs1, Chk2 and p53 (Lilley, Carson et al. 2005; Shirata, Kudoh et al. 2005; Li, Baskaran et al. 2008), however ICP0 mediates the degradation of RNF8 and RNF168 (Lilley, Chaurushiya et al. 2010), which are key mediators in this DDR pathway. Therefore, this suggests that HSV-1 has evolved to modulate components of the same DDR pathway temporally and differentially to ensure its own replication. Given that BRCA1 is an important downstream target in the ATM-dependent pathway, our result was intriguing because while proteins repressive for the viral infection seem to be degraded, proteins that facilitate the infection are retained (Lilley, Chaurushiya et al. 2011). Therefore, as this unknown protein could be advantageous for the virus a more detailed characterization of the 120-kDa protein revealed by a BRCA1 specific antibody on western blot was undertaken.

2. Study of the 120 KDa-protein, reactive with an antibody specific for BRCA1, induced after HSV-1 infection:

2.1. Analysis of the induction of the 120 KDa-protein after viral infection of a range of cell lines.

Given that we had observed a marked increase in the levels of a potential BRCA1 variant in HFF and U2OS cells infected with HSV-1 (Fig. R9), it was of interest to ascertain whether this result was consistent in a range of cells susceptible to infection by HSV-1 or whether this response was specific to HFF and U2OS cells. To address this question, the breast epithelial cancer cell lines, MCF7 and MDA-MB-231, were infected with the control revertant HSV-1 (*dl*1403R) or with vaccinia virus (VACV, another DNA virus belonging to the poxvirus family) at m.o.i.s of 10. These experiments showed that infection with HSV-1, but not VACV, led to an upregulation of the 120 kDa-protein in MCF7 and MDA-MB-231 cells (Fig. R10-A). Both infected cell lines were collected at 24 hpi because HSV-1 and VACV have similar kinetics of infection (Fields 1990). The absence of upregulation of the 120 KDa-protein in HFFs after VACV infection was confirmed in experiments where HFFs were infected with HSV-1 (*dl*1403R) and VACV at m.o.i. 10 (Fig. R10-B). Consistent with the previous data, HFFs infected with VACV did not show an increase in the expression of the 120 KDa-protein.

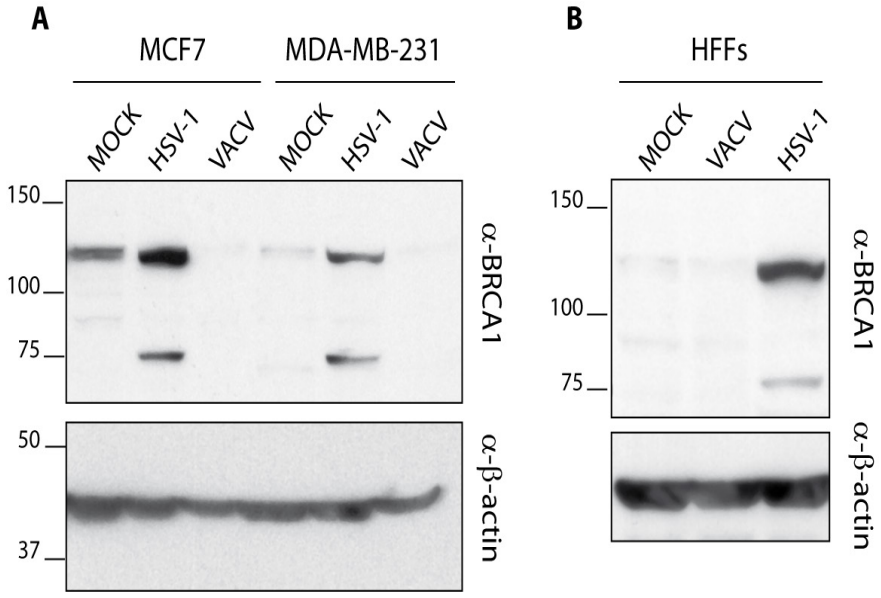


Figure R10. HSV-1 infection, but not VACV infection, induces a marked increase in the levels of a 120-KDa protein reactive with an antibody specific for BRCA1 in MCF-7, MDA-MB-231 and HFF cells. (A) MCF-7 and MDA-MB-231 cells were infected with either HSV-1 (dl1403R) or VACV (WR strain) at 10 pfu/cell. Total cell lysates were prepared at 24 hpi and 50 µg of total protein were analysed by western blot with antibodies to BRCA1 (I-20) and β-actin. (**B)** HFF cells were infected with either HSV-1 (dl1403R) or VACV (WR strain) at 10 pfu/cell. Total cell lysates were prepared at 24 hpi and 50 µg of total protein were analysed by western blot with antibodies to BRCA1 (I-20) and β-actin.

Although VACV is a large DNA virus, like HSV-1, VACV replicates in the cytoplasm of host cells whereas HSV-1 replicates in the nucleus. To test whether this difference could explain the absence of the 120 KDa-protein after VACV infection, HFFs were infected with human cytomegalovirus (HCMV), a β herpesvirus that also replicates in the nucleus. Since HCMV replicates with slower kinetics than HSV-1 (Fields 1990), HFFs infected with HCMV at m.o.i. of 10 were harvested at 48 h.p.i. We found that HCMV infection did not induce the expression of the 120 KDa-protein, confirming that the appearance of this protein was specific for HSV-1 infection (Fig. R11).

A time course experiment was also performed to analyse the kinetics of the induction of expression of the 120 KDa-protein after HSV-1 expression, because in previous experiments the expression of this protein was only analysed at 24 h.p.i. HSV-1 (*dl*1403R) infection was done using 10 pfu/cell (Fig. R11). This time course experiment revealed that upregulation of the 120 kDa-protein was first detected at 4 hours of HSV-1 lytic infection and increased as HSV-1 infection proceeded (Fig. R11).

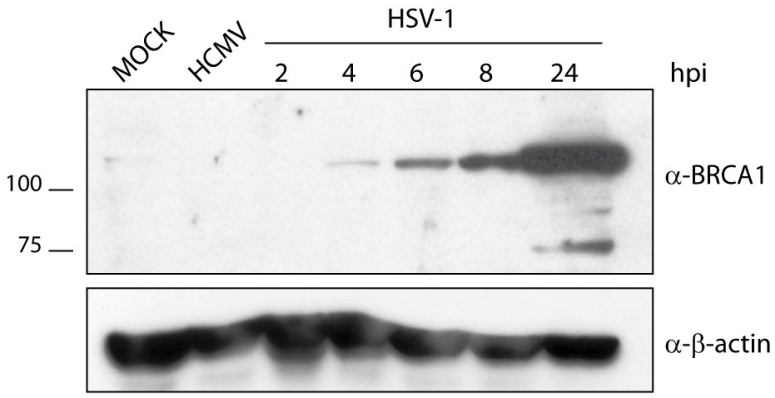


Figure R11. HCMV does not induce the 120 KDa-protein expression and its upregulation during HSV-1 infection increases as infection progresses in HFF cells. HFF cells were infected with HCMV (strain AD169) at 10 pfu/cell and harvested at 48 hpi. HHF cells were infected with HSV-1 at 10 pfu/cell and the expression of the 120 KDa-protein was monitored over the time. 50 µg of total protein were analysed by western blot with antibodies to BRCA1 (I-20) and β-actin.

From these data it can be concluded that the upregulation of the 120 KDa-protein is specific to HSV-1 infection because, at least VACV and HCMV infections did not induce expression of this protein. Although an extensive cytopathic effect was observed when infected cells were collected, a western blot control for VACV and HCMV infection would have been a better control for efficient infection with both viruses. Furthermore, the upregulation occurred in all the cells lines analysed.

It is important to note that in many of the western blot analyses, a weak band around 80 KDa appeared together with the 120 KDa band in the lane corresponding to the HSV-1-infected cells. But we decided to focus on the strong 120 KDa-protein, which was consistently expressed. Moreover, a

very weak band (more intense in MCF7 cells) can be seen in the mock cells but its molecular weight was a bit higher than the 120 KDa band observed on infected cells. One possibility to explain that was that the band on mock cells was the same 120 KDa protein but at a different post-translational modification status.

2.2. Evaluation of the possible involvement of viral genes in the upregulation of the 120 KDa-protein during HSV-1 infection:

Given that preliminary experiments showed that ICP0 is not a key factor in the increase of the 120 KDa-protein levels (Fig. R9), a different panel of HSV-1 mutant viruses (Table R2) was used to study which viral proteins could have a role in the upregulation of the 120 KDa-protein.

VP16 is a tegument protein, released into host cell upon virus entry, that is essential for efficient viral replication because it is the transcriptional activator of the IE genes (Campbell, Palfreyman et al. 1984) and that also plays a role during virion assembly (Ace, Dalrymple et al. 1988; Weinheimer, Boyd et al. 1992). To assay whether VP16 was involved in induction of the 120 KDa-protein, HFFs were infected with the HSV-1 mutant in1814 (derived from HSV-1 strain 17) (Table R2), which contains a 12-base-pair insertion in the gene encoding VP16 that compromises its transactivating function (Ace, McKee et al. 1989) (Fig. R12).

HFFs were also infected with a mutant deficient in the viral glycoprotein E (ΔgE) (Table R2) to check if this mutant could also provoke the upregulation of the 120 KDa-protein (Fig. R12). This mutant was tested because we had planned to do immunofluorescence experiments to study the localization of the 120 KDa-protein and the complex of the viral glycoproteins gE/gI is well known to form a receptor for the Fc domain of immunoglobulin G (IgG) (Baucke and Spear 1979; Johnson and Feenstra 1987; Johnson, Frame et al. 1988). This Fc-receptor (FcR) has a strong capacity to bind human IgGs, decreasing affinity for rabbit, goat and sheep and not affinity for murine IgGs (reviewed in (Dubin, Fishman et al. 1992)). Therefore, to avoid non-specific signals due to the binding of gE/gI to the Fc portion of the rabbit and goat primary and secondary antibodies, it was convenient to use a virus deficient in gE. The mutant in gE was preferred over an HSV-1 mutant lacking gI alone, because gE alone can bind IgG (although with lower affinity than the Fc binding observed with gE and gI together) whereas gI alone does not bind IgG (Bell, Cranage et al. 1990; Dubin, Frank et al. 1990; Hanke, Graham et al. 1990).

Furthermore, to test whether the observed band was induced by an unexpected effect of the BHK (baby hamster kidney cells) debris present in the viral stocks, HFFs were exposed to concentrated supernatants from uninfected BHKs and were also infected with purified HSV-1 viruses (MP, medium-purified viruses) in addition to the cell-associated viruses (CA) (Fig. R12).

Lysates prepared from HFFs 24 h.p.i with wt (strain 17) or the different mutant HSV-1 virus at a m.o.i. of 20, were analysed by western blot with the previously used rabbit polyclonal anti-BRCA1 antibody

(Santa Cruz I-20) together with a mouse monoclonal antibody anti-gD (LP14) as a control of infection and a mouse monoclonal antibody anti-actin as a loading control (Fig. R12).

Mutant	Protein mutated	Description
ΔgE	**gE**	LacZ insertion within the gene encoding gE (US8)
*in*1814	**VP16**	12-base-pair insertion in the gene encoding VP16
tsk	**ICP4**	Temperature sensitive mutation in the gene encoding ICP4
*in*1382	**VP16** **ICP4** **ICP0**	12-base-pair insertion in the gene encoding VP16 Temperature sensitive mutation in the gene encoding ICP4 Deletion within the RING finger of ICP0

Table R2. HSV-1 mutants used in this study.

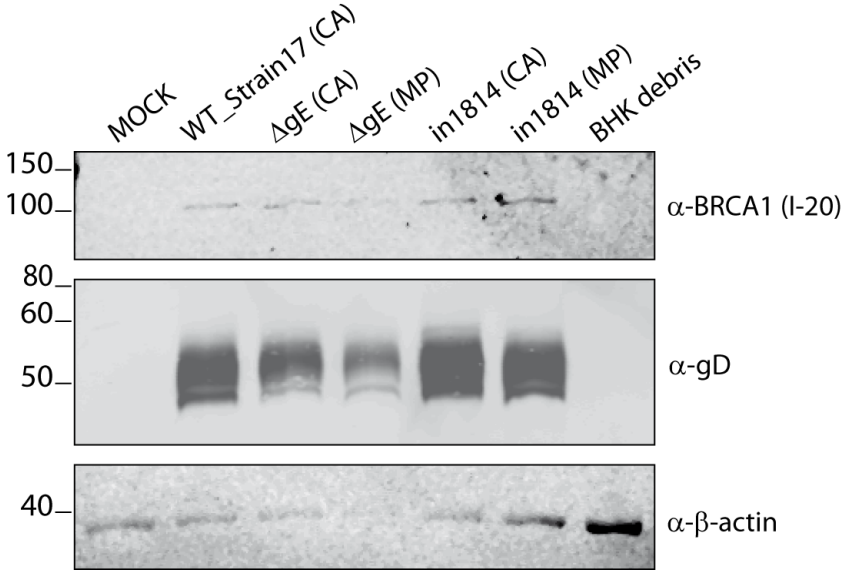


Figure R12. Infection with wt HSV-1 and different HSV-1 mutants induces an increase in the levels of the 120-KDa protein reactive with the anti-BRCA1 antibody I-20. HFF cells were infected with wt HSV-1 (strain 17 CA stock), the mutant deficient in gE (strain SC16 CA and MP stocks) and the mutant in1814 (strain 17 CA and MP stocks) at 20 pfu/cell. HFFs were also exposed to BHK debris. Total cell lysates were prepared at 24 hpi and were analysed by western blot with antibodies to BRCA1 (I-20), gD and β-actin.

As shown in Fig. R12, infection with all the different HSV-1 viruses, either cell-associated (CA) or medium-purified stocks (MP), led to the upregulation of the 120 KDa-protein, in contrast no increased expression of this protein was noted in HFFs exposed to BHK debris. The different conditions used for the western blot analysis (primary antibodies were incubated with 5% skimmed milk 1h at RT, for more specifications see Materials and Methods) might explain why the 120 kDa band was less marked.

To further explore these data, HFFs were next infected with the mutant tsK (isolated from

HSV-1 strain 17+) (Table R2) at m.o.i. 20 (Fig. R13). This mutant carries a temperature sensitive mutation in the DNA binding domain of ICP4, allowing virus replication to proceed at 31°C but not at 37-38°C (Preston 1979; Davison, Preston et al. 1984). HFFs infected with this virus were incubated at 37°C and harvested 24 h.p.i. Lysates prepared from these cells were analysed by western blot using the antibody against BRCA1 (Santa Cruz I-20).

This experiment showed that even in the absence of functional ICP4 the band of 120 KDa still appeared (Fig. R13), although the induction was less marked. As might be expected, gD staining showed that infections with the ICP4 mutant tsK seem to progress less efficiently than with wt strain 17 virus.

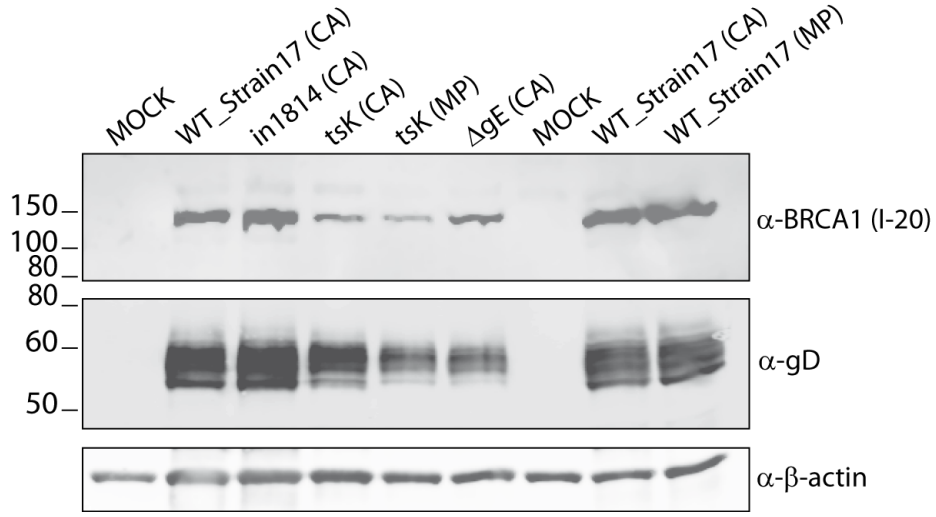


Figure R13. Infection with different mutants of HSV-1 induces expression of the 120-KDa protein reactive with the anti-BRCA1 antibody I-20. HFF cells were infected with wt HSV-1 (strain 17 CA and MP stock), the mutant in1814 (CA), the mutant deficient in gE (CA stock) and the mutant TSK (CA and MP stocks) at 20 pfu/cell. Total cell lysates were prepared at 24 hpi and were analysed by western blot with antibodies to BRCA1 (I-20), gD and β-actin.

It is important to note that previous publications showed that the defect in IE gene transactivation of the mutant *in*1814 was observed at low m.o.i., however, IE gene transinduction by VP16 was not essential for viral growth at high m.o.i. (at m.o.i. 1 the viral gene expression in *in*1814 relative to that observed in wt HSV-1 was reduced by 33-fold, at m.o.i. 10 it was reduced by 6-fold but in1814 was not detectably impaired at m.o.i. 100) (Ace, McKee et al. 1989). Since we infected at m.o.i. 20, IE gene expression, and hence the viral growth should not be severely affected (as reflected by gD staining in Figs. R12 and R13).

Moreover, the mutant tsk carries a lesion in ICP4 that affects its normal functions at non-permissive temperatures, including the capacity of ICP4 to repress IE gene expression, thus, in infection with tsk virus the other IE proteins are over-produced (Watson and Clements 1978; Preston 1979; DeLuca, McCarthy et al. 1985).

Therefore to reduce further viral gene expression, HFFs were infected with the mutant *in*1382 (Preston

and McFarlane 1998; Preston, Rinaldi et al. 1998), which contains the insertion in the VP16 coding sequence derived from *in*1814, the temperature sensitive mutation in the ICP4 coding sequence derived from tsK and additionally, a deletion within the RING finger domain of ICP0 rendering it non-functional (Table R2) (Fig. R14). Furthermore, to further study if this effect was specific to HSV-1, HFFs were infected with HSV-2, another member of the genus *Simplexvirus* which is closely related to HSV-1 (Fig. R14). In this experiment all the infections were carried out at m.o.i. of 3 instead of the previously used of 10-20 in order to study if the same result is observed at lower multiplicities, because as stated previously when cells are infected with low number of these mutant viruses (with mutations in VP16 or ICP0), viral replication and gene expression are greatly reduced comparing to wt infections (Ace, McKee et al. 1989; Everett 1989).
All the infected HFFs were incubated at 37 °C and harvested 24 h.p.i. Lysates prepared from these HFFs were analysed by western blot using the antibody against BRCA1 (Santa Cruz I-20).

Cells infected with *in*1382 could induce expression of the 120 KDa-protein, but at much lower levels than that observed for the wt or ΔgE viruses (Fig. R14). Infection with the wt and ΔgE viruses triggered the increased expression of the 120 KDa-protein levels even at low m.o.i., confirming the previous experiments and showing that this effect was not m.o.i.-dependent. Surprisingly, the levels of gD detected in cells infected with the *in*1382 virus were not very different from those observed after infection with the other HSV-1 viruses used. Given that this mutant, *in*1382, has difficulties in initiating a lytic cycle of viral replication due to the absence of functional VP16, ICP4 and ICP0, the three major transactivator proteins, it is possible that the gD detected in these experiments represents glycoprotein persisting from the input virus used for infection. Infection with HSV-2 was not associated with the induction of expression of the 120 KDa-protein (Fig. R14).

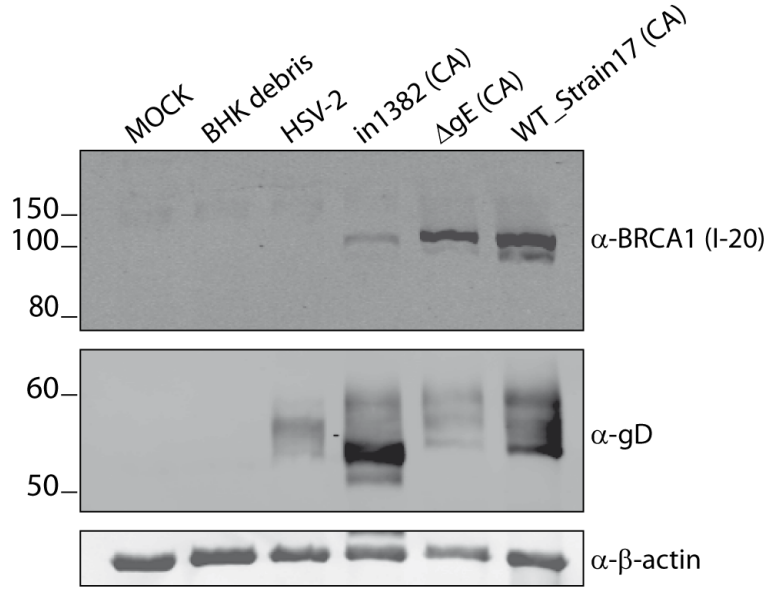


Figure R14. Infection with different mutants of HSV-1 induces the increase in the levels of the 120-KDa protein reactive with the anti-BRCA1 antibody I-20. HFF cells were infected with wt HSV-1 (strain 17 CA), the mutant in1382 (CA), the mutant deficient in gE (CA stock) and HSV-2 (CA stock) at 3 pfu/cell. HFFs were also exposed to BHK debris. Total cell lysates were prepared at 24 hpi and were analysed by western blot with antibodies to BRCA1 (I-20), gD and β-actin.

Altogether these data (Figs. R12-13 and 14) showed that infection with all the different HSV-1 mutants, either as cell-associated (CA) or medium-purified stocks (MP), triggered upregulation of the 120-kDa protein. In contrast, neither exposure to BHK debris, nor infection with HSV-2 virus led to expression of the 120 kDa-protein. Therefore it can be concluded that the presence of the 120 kDa band is specific for HSV-1 infection.

2.3. Assessment of the reactivity of the N-terminal D20 BRCA1 specific antibody with the 120 KDa-protein.

The band observed after HSV-1 infection with the BRCA1 I-20 antibody has a molecular weight of 120 KDa approximately; however, the size of the canonical sequence of BRCA1 protein in SDS-PAGE is 220-230 KDa. Multiple different splice variants of BRCA1 have been described (Lu, Conzen et al. 1996; Thakur, Zhang et al. 1997; Wilson, Payton et al. 1997; Xu, Chambers et al. 1997), but none of them is detected at 120 KDa. However, a BRCA1 variant lacking most of exon 11 (BRCA1-Δexon11b) seems to encode a protein of 110 kDa (Wilson, Payton et al. 1997). Moreover the band of approximately 220 KDa corresponding to the full length molecule was not observed in our experiments with the I-20 antibody, raised by immunization with a specific peptide of the C-terminal region of the human BRCA1.
Given this discrepancy between the consensus molecular weights of BRCA1 species and our observations, another antibody against BRCA1 was used to confirm the upregulation of this protein following HSV-1 infection, and discard any possible I-20 antibody crossreactivity. Given that the antibody I-20 used was raised against a peptide mapping near the C-terminus of human BRCA1, we used an antibody designed against the N-terminus of the molecule: the rabbit polyclonal D-20 antibody (Santa Cruz Biotechnology).

Some of the lysates used in the previous experiments (Figs. R12, 13 and 14) were subjected to western blotting using the N-terminal D-20 antibody (Figs. R15, 16 and 17). This antibody recognized mainly a protein of about 80-90 KDa in HSV-1 infected cells, but it did not detect the previously seen 120 KDa protein or the canonical 220 KDa form of BRCA1. Moreover Figure R17 also showed that D-20 antibody detected numerous bands from 60 to 120 kDa approximately even in uninfected cells, cell exposed to BHK debris and HSV-2 infected cells.

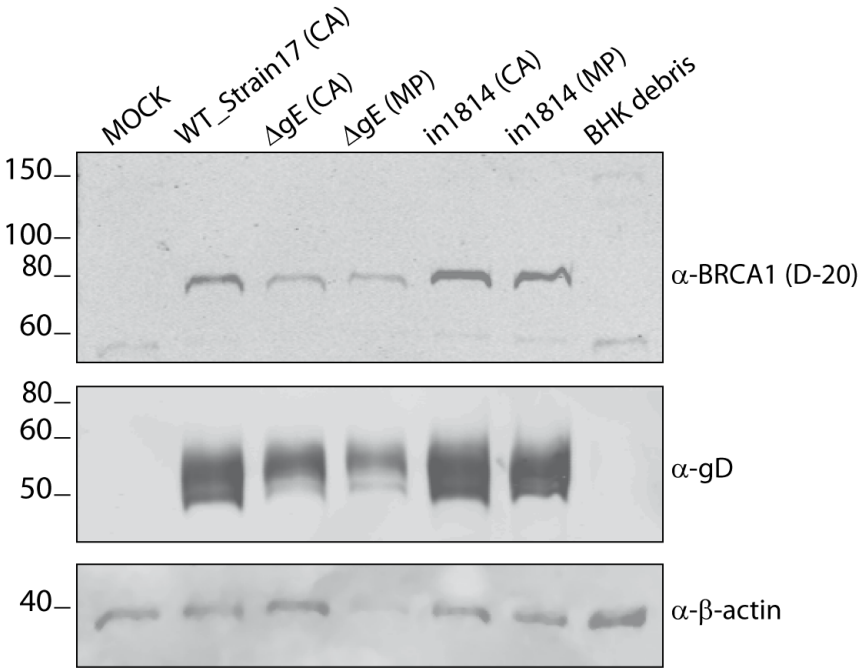


Figure R15. Infection with different mutants of HSV-1 induces an increase in the levels of an 80-KDa protein reactive with the N-terminus anti-BRCA1 antibody D-20. HFF cells were infected with wt HSV-1 (strain 17 CA stock), the mutant deficient in gE (strain SC16 CA and MP stocks) and the mutant in1814 (CA and MP stocks) at 20 pfu/cell. HFFs were also exposed to BHK debris. Total cell lysates were prepared at 24 hpi and were analysed by western blot with antibodies to BRCA1 (D-20), gD and β-actin.

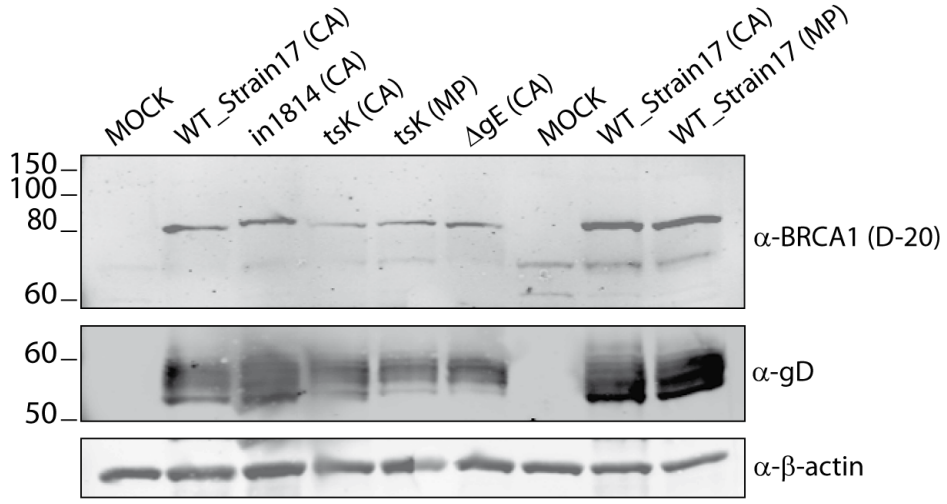


Figure R16. Infection with different mutants of HSV-1 induces an increase in the levels of an 80-KDa protein reactive with the N-terminus anti-BRCA1 antibody D-20. HFF cells were infected with wt HSV-1 (strain 17 CA and MP stock), the mutant in1814 (CA), the mutant deficient in gE (CA stock) and the mutant TSK (CA and MP stocks) at 20 pfu/cell. Total cell lysates were prepared at 24 hpi and were analysed by western blot with antibodies to BRCA1 (D-20), gD and β-actin.

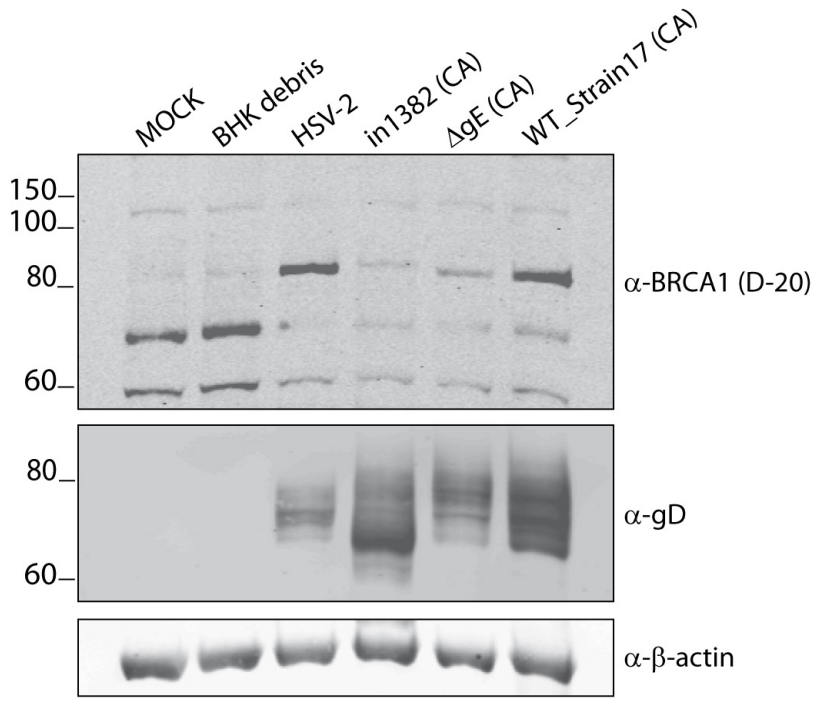


Figure R17. Infection with different mutants of HSV-1 induces an increase in the levels of an 80-KDa protein reactive with the N-terminus anti-BRCA1 antibody D-20. HFF cells were infected with wt HSV-1 (strain 17 CA), the mutant in1382 (CA), the mutant deficient in gE (CA stock) and HSV-2 (CA stock) at 3 pfu/cell. HFFs were also exposed to BHK debris. Total cell lysates were prepared at 24 hpi and were analysed by western blot with antibodies to BRCA1 (D-20), gD and β-actin.

In our experiments the I-20 and D-20 antibodies detected different sets of proteins induced after HSV-1 infection, but not a protein that can be unambiguously identified as the canonical form of BRCA1. However, it is well known that it is difficult to detect endogenous BRCA1 expression using immunoprecipitation and western blot analysis, as described below. Therefore, the presence of the bands observed not at the proper molecular weight could represent the same target with different post-translational modifications, splice variants, breakdown products, or different proteins.

2.4. Study about BRCA1 antibodies in the literature:

In the light of these results, an extended literature research about antibodies against BRCA1 was performed, and a controversy in this field was found, indicating a lack of antibodies with the required specificity:

In the middle of the nineties, some reports using the commercially available C-20 antibody (Santa Cruz Biotechnology) showed that *BRCA1* encodes a 180-190 KDa protein (Gudas, Nguyen et al. 1995) with sequence homology and biochemical analogy to the granin family of secretory proteins. Therefore BRCA1 was considered to be secreted from breast epithelial cells (Jensen, Thompson et al. 1996). However, it was later concluded by Wilson *et al.* in 1996 that the C-20 antibody cross-reacted with the epidermal growth factor receptor (EGFR, also called HER1), which migrated in SDS-PAGE with a size about 190 kDa, and the human epidermal growth factor receptor 2 (HER2), which migrated at 205 kDa approximately (Wilson, Payton et al. 1996). Later in the same year another publication reported that the C-20 antibody, in addition to the 220 KDa protein, also recognized a 180

Kda protein in total lysates from human breast cancer cell line MDA-MB-468 that was no detected by other antibodies against BRCA1. And they suggested that this protein was EGFR (Thomas, Smith et al. 1996). Next year an additional report confirmed the cross-reactivity of the C-20 antibody to the EGFR and they also reported cross-reaction of this antibody with EGF (Bernard-Gallon, Crespin et al. 1997). One year later a new report showed another cross-reactivity of the C-20 antibody because it detected smooth muscle cells, although the exact epitope that this antibody recognized is not clear yet (Imakado, Hoashi et al. 1998).
The C-20 antibody is raised against residues 1843-1862 at the carboxy terminus of the BRCA1 protein. The aminoacid sequence alignment between the peptide used to generate this antibody and the members of the type I tyrosine kinase receptor family revealed a similarity with EGFR consisting of six identical amino acids, including the tyrosine autophosphorylation site at position 992 of EGFR, as well as three conservative substitutions. Similar matches were observed with the homologous autophosphorylation sites in HER2 and HER4 (Wilson, Payton et al. 1996).

The I-20 antibody (Santa Cruz Biotechnology) that we had been using is also a C-terminal antibody, but it is raised against residues 1823-1842. Although I-20 did not cross-react with type I receptor tyrosine kinase receptors (Wilson, Payton et al. 1996) and it did not stain smooth muscle cells as did C-20 (Imakado, Hoashi et al. 1998), it was found by Wilson and coworkers (the same group that discovered the crossreaction of the C-20) that in cytoplasmic, nuclear and total cell extracts from simian Cos-7 kidney cells transfected with full length BRCA1, the I-20 antibody recognized, besides the 230 KDa over-expressed protein, an unknown abundant, predominantly cytoplasmic protein slightly smaller than BRCA1. It was suggested that this protein was not BRCA1 because it was not detected with other antibodies against BRCA1 (Wilson, Payton et al. 1997).
Wilson and colleagues further evaluated the specificity and utility of 19 antibodies against different epitopes of BRCA1 and revealed that the C-20, D-20 and I-20 antibodies recognized additional proteins in human SK-OV3 extracts, which were probably not products of alternative splicing because they were not observed with other antibodies against overlapping BRCA1 sequences. Furthermore, the I-20 was the only antibody that was unable to identify the BRCA1 220 kDa protein in both human epithelial HBL-100 and SK-OV3 cell lines extracts (Wilson, Ramos et al. 1999). The same year another report suggested that some C-terminal antibodies, among them C-20 and I-20, cross-reacted with a protein(s) different from BRCA1 because they showed false-positive immunostaining in *BRCA1*-associated cancer cells that had a *BRCA1* protein-truncating mutation and in *BRCA1*-deficient cell line HCC1937 that lacks the C-terminus of the BRCA1 protein (Yoshikawa, Honda et al. 1999). The specificity of I-20 was questioned again years later since this antibody showed predominantly cytoplasmic staining in immunohistochemical analyses with no differences in staining between tumoral and non-tumoral mammary epithelial cells as would be expected (Perez-Valles, Martorell-Cebollada et al. 2001). Moreover, another study discarded I-20 and also C-20 antibodies for further analysis of BRCA1 expression due to the non-specificity of these antibodies in western blot and immunohistochemical

staining controls (Bogdani, Teugels et al. 2002).

Therefore, the C-terminal antibodies, such as C-20 and I-20, were found to produce non-specific additional staining patterns. With respect to the I-20 antibody, it has been reported that this antibody may recognize other proteins besides BRCA1 or products of alternative splicing of the gene, thus, the possibility that the results obtained using the I-20 antibody were influenced by cross-reactive binding to non BRCA1 proteins was carefully evaluated.

Firstly, the possibility that the protein around 120 KDa recognized by the I-20 antibody was some form of EGFR was tested, since the predicted molecular weight of EGFR is 134 KDa, although it is usually detected around 180 KDa in western blot analysis due to its post-translational modifications. To address this issue, lysates from two cell lines with different levels of EGFR expression, HFF and MCF7, mock infected and infected with HSV-1 were subjected to western blot analysis using an antibody specific for EGFR together with the I-20 antibody (Fig. R18). We used these cell lines because it is known that fibroblasts express EGFR (40,000-100,000 receptors/cell) (Hollenberg and Cuatrecasas 1973; Carpenter, Lembach et al. 1975; Hollenberg and Cuatrecasas 1975) while MCF-7 have low levels of EGFR expression (~4,000 receptors/cell) (Davidson, Gelmann et al. 1987; Goldenberg, Masui et al. 1989; Kaplan, Jaroszewski et al. 1990; Kute and Quadri 1991; Sharma, Horgan et al. 1994).

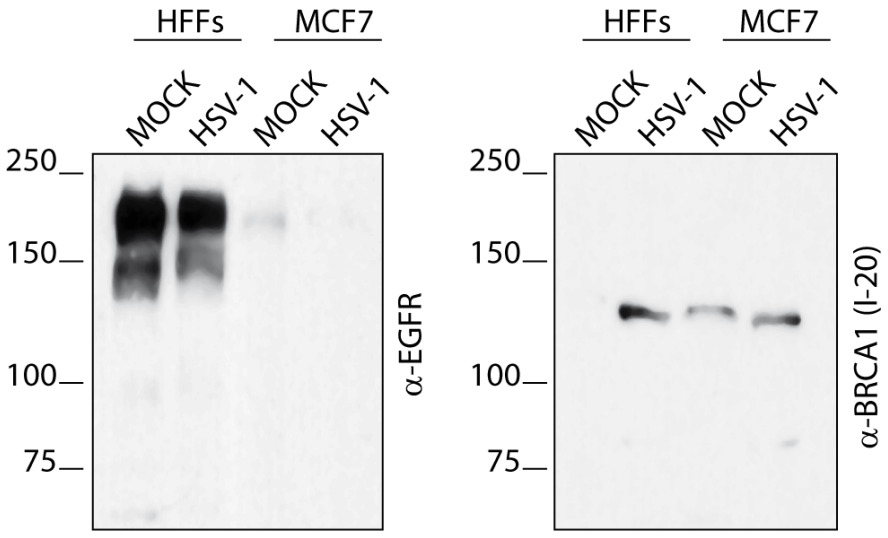


Figure R18. The antibody anti-BRCA1 I-20 does not cross-react with EGFR. HFF and MCF-7 cells were infected with HSV-1 (dl1403R) at 10 pfu/cell. Total cell lysates were prepared at 24 hpi and 30 µg were analysed by western blot with antibodies to EGFR and BRCA1 (I-20).

Our result showed that EGFR banding pattern was completely different from that observed with I-20, indicating that the band around 120 KDa did not correspond to EGFR. Therefore we confirmed previous findings showing that immunoblot analysis with an EGFR antibody of I-20 immunoprecipitates did not detect EGFR (Wilson, Payton et al. 1996).

Given that our observations raised concern about the identity of the proteins recognized by the I-20 antibody, lysates from infected HFF cells were subjected to immunoprecipitation with the I-20 antibody for subsequent analysis of the immunoprecipitated proteins by mass spectrometry to resolve the identity of the proteins recognized in western blot analysis, specifically the most intense band around 120 KDa (section 2.6). While proteomic analysiswas underway, we examined in parallel another hypothesis about the identity of the 120 KDa-protein. The pattern of bands observed could be product of a BRCA1 cleavage during HSV-1 infection. If the protein was cleaved, the epitopes of the I-20 and D-20 antibodies might become more accessible; thereby, the N-terminus fragment around 90 KDa could be recognized by the D-20 antibody and the C-terminal fragment around 120 KDa could be recognized by the I-20 antibody (section 2.5).

2.5. Study of a possible cleavage in full-length BRCA1 during HSV-1 infection.

If HSV-1 infection led to a cleavage of full-length BRCA1 protein, the resulting BRCA1 fragments could be predicted to have different patterns of subcellular localization. Given the distribution of the nuclear localization signals (NLS) and the RING finger domain of BRCA1, the N-terminal fragment around 90 Kda, recognized by D-20 antibody, would be predicted to be retained in the nucleus while the C-terminal fragment of approximately 120 KDa, recognized by I-20 antibody, would be expected to be located in the cytoplasm (Fig. R19).

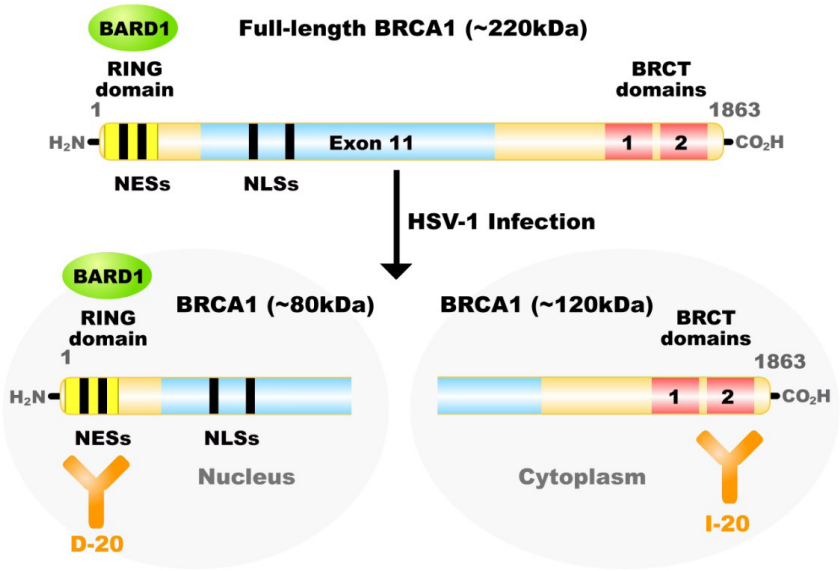


Figure R19. Schematic representation of the hypothetical cleavage of full-length BRCA1 mediated by HSV-1. BRCA1 contains two main functional domains, a RING finger domain and the BRCA1 C-terminal (BRCT) domains. The RING domain, responsible for BARD1 binding, the nuclear export signals (NESs), the exon 11, the nuclear localization signals (NLSs) and the BRCT domains are indicated. If HSV-1 infection led to a cleavage of full-length BRCA1 protein, the resulting BRCA1 fragments around 80 and 120 kDa, recognized by D-20 and I-20 antibodies respectively, would be expected to be retained in the nucleus and the cytoplasm respectively.

The subcellular distribution of BRCA1 has been studied by numerous groups, however there are many discrepancies between these different studies in the literature.

Generally, BRCA1 has been considered to be a predominantly nuclear protein involved in multiple nuclear located functions. BRCA1 has two nuclear localization signals (NLS1 and NLS2) located within exon 11, the largest of the exons in the BRCA1 gene. Two mechanisms for BRCA1 nuclear import have been described; the classical importin α/β pathway, in which NLS1 interacts directly with importin α, which in turn interacts with importin β. And the second pathway involves a NLS-independent mechanism, in which BRCA1 binds to BRCA1-associated RING domain protein 1 (BARD1) via their RING finger domains, and then this BRCA1:BARD1 complex binds to the importin machinery interacting first with importin α. However, BRCA1 can also be exported from the nucleus. BRCA1 has two nuclear export signals (NES), at amino acid residues 81-99 and 22-30, which bind to the nuclear export receptor CRM1 (chromosome region maintenance 1; also known as exportin1) to translocate BRCA1 out of the nucleus. Therefore BRCA1 can shuttle between the nucleus and the cytoplasm. However, it has been suggested that the interaction of BRCA1 with BARD1 inhibits the nuclear export of BRCA1 because BARD1 masks the BRCA1 NESs, since these sequences are located within the RING finger domain of BRCA1 (aa 1-109). All these observations indicate an important role of BARD1 for both nuclear import and export of BRCA1 and in aggregate explain why BRCA1 is predominantly detected in the nucleus (reviewed in (Thompson 2010)).

In order to explore the hypothesis of HSV-1-induced cleavage of BRCA1, localization studies by immunofluorescence analysis were carried out. HFF cells were mock infected or infected with ΔgE HSV-1 using the previously used m.o.i of 20 and subjected to immunofluorescence analysis 7-8 h.p.i using different anti-BRCA1 antibodies.

The cells were infected with the ΔgE virus in order to avoid non-specific signal due to the binding of gI/gE to the Fc portion of the primary and secondary antibodies, as explained before. And they were fixed 7-8 h.p.i because we observed in the time course that the marked increase in expression of the 120 kDa-protein was clear 6 h.p.i (Fig. R11) and at this time the cells are not massively damaged due to the infection.

The panel of anti-BRCA1 antibodies comprised the previous C-terminal I-20 (rabbit polyclonal Santa Cruz Biotechnology) and the N-terminal D-20 (rabbit polyclonal Santa Cruz Biotechnology) antibodies; as well as a mouse monoclonal antibody specific for BRCA1, MS110 (Millipore), raised against the N-terminus.

Since the I-20 and D-20 antibodies have been suggested to cross-react with proteins other than BRCA-1 (Wilson, Payton et al. 1997; Wilson, Ramos et al. 1999; Yoshikawa, Honda et al. 1999), we also used the MS110 antibody, also called Ab-1 in the literature and generated by the Livingston's group (Scully, Ganesan et al. 1996), to corroborate our previous results. This N-terminal antibody has been widely used for detection of BRCA1 and has been considered one of the best antibodies for BRCA1

detection, even though previous studies also indicated potential non-BRCA1 reactivities of the MS110 antibody (Yoshikawa, Honda et al. 1999; Perez-Valles, Martorell-Cebollada et al. 2001; Fraser, Reeves et al. 2003; Mangia, Chiriatti et al. 2009). Specifically, recent mass spectrometry analyses have shown that MS110 clearly binds two proteins different from BRCA1, Sec16A and PCM1, in lysates of HeLa cells. Moreover, three other bands of lower molecular size were also identified as possible additional non-specific interactions (Milner, Wombwell et al. 2013). In spite of these data, this antibody has been considered to produce the most reliable and consistent results mainly in immunohistochemical analysis (Wilson, Ramos et al. 1999; Yoshikawa, Honda et al. 1999; Perez-Valles, Martorell-Cebollada et al. 2001; Al-Mulla, Abdulrahman et al. 2005; Alamshah, Springall et al. 2008; Milner, Wombwell et al. 2013).

As shown in Fig. R20 ΔgE HSV-1 infection of HFF cells led to a marked increase in staining with I-20 and this signal was homogeneous in intensity in both nucleus and cytosol. Moreover, the immunofluorescence staining intensity directly correlated with the amount of the 120 KDa-protein detected by our western blot analyses.

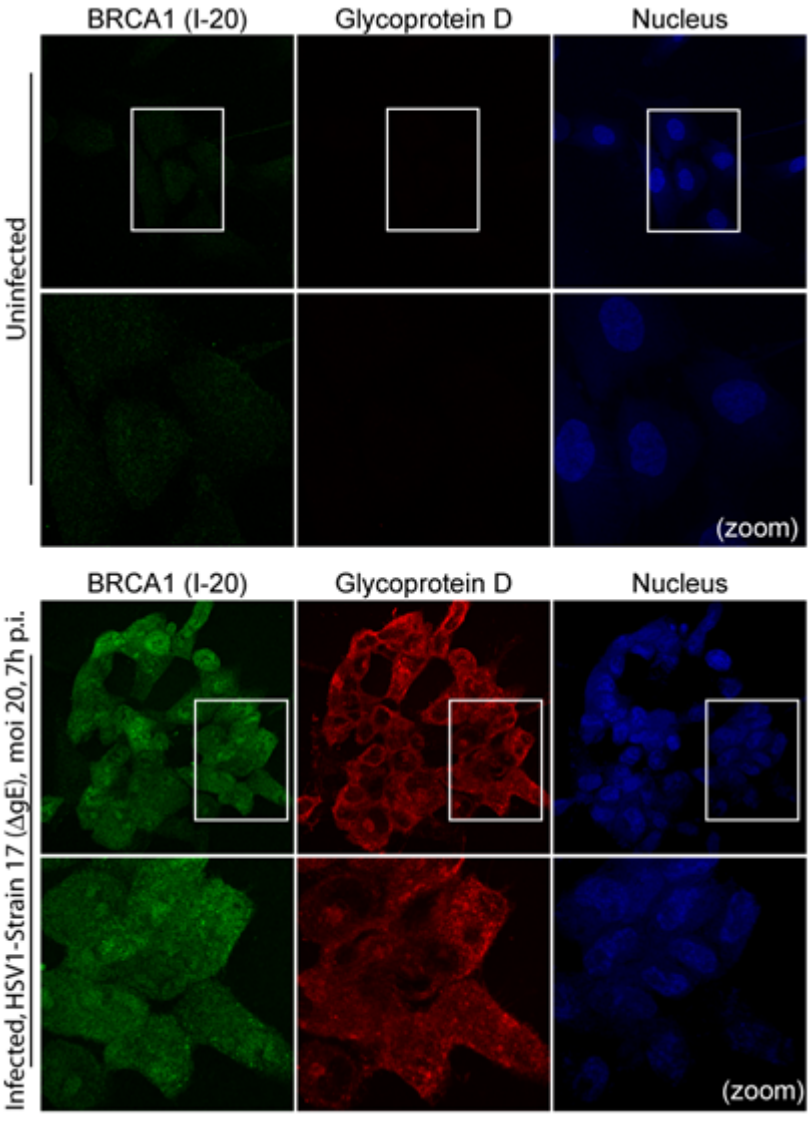


Figure R20. Staining with the I-20 antibody shows an increased signal, in both nucleus and cytosol, after HSV-1 infection of HFF cells. HFF cells were mock or infected with ΔgE (20 p.f.u/cell). 7 h.p.i uninfected and infected cells were fixed in 2% paraformaldehyde and stained with anti-BRCA1 I-20 plus goat anti-rabbit Alexa Fluor 488 (green, left panel) and anti-gD LP2 plus donkey anti-mouse Alexa Fluor 568 (red, middle panel) as a control of infection. Nuclei were stained with DAPI (blue, right panel).

Staining with the D-20 antibody showed no differences in intensity or localization between non-infected and infected cells, with signal observed in both the cytoplasm and nucleus (Fig. R21).

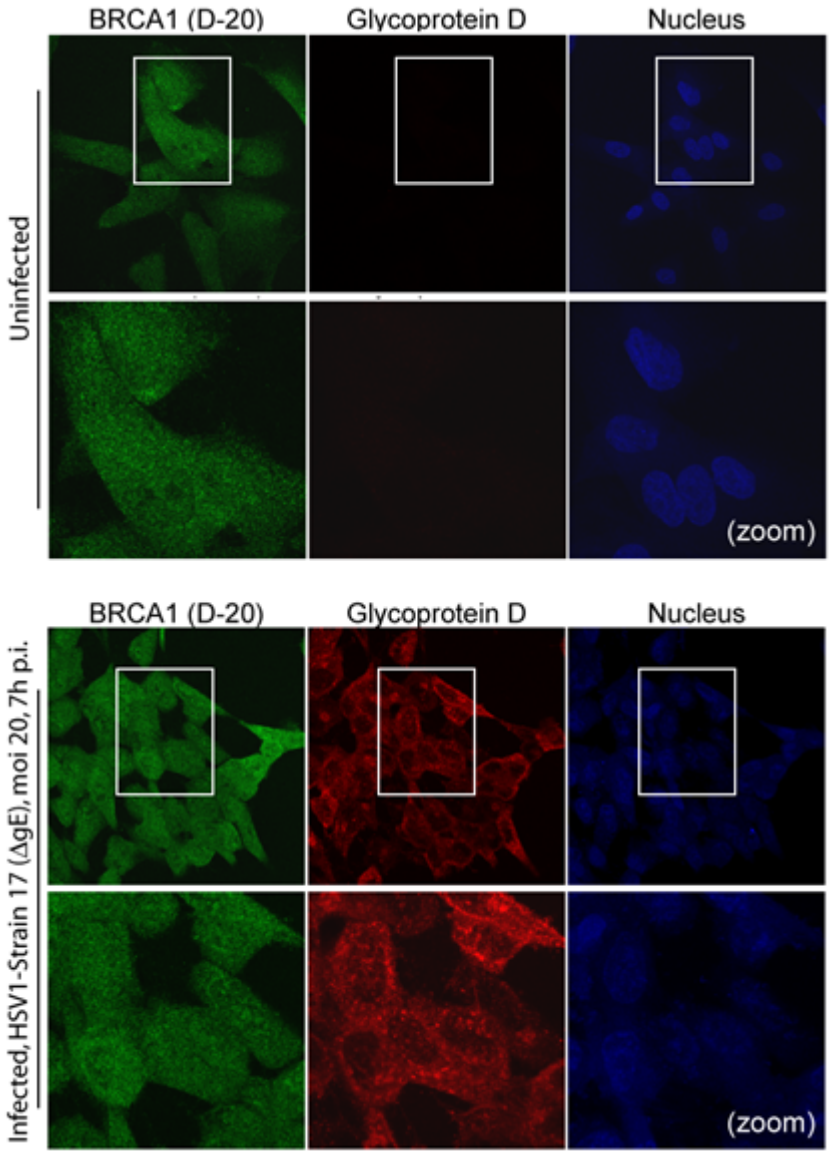


Figure R21. Staining with the D-20 antibody shows no differences between noninfected and infected HFF cells. HFF cells were mock or infected with ΔgE (20 p.f.u/cell). 7 h.p.i uninfected and infected cells were fixed in 2% paraformaldehyde and stained with anti-BRCA1 D-20 plus goat anti-rabbit Alexa Fluor 488 (green, left panel) and anti-gD LP2 plus donkey anti-mouse Alexa Fluor 568 (red, middle panel) as a control of infection. Nuclei were stained with DAPI (blue, right panel).

Since the marked cell damage produced by infection using an m.o.i. of 20 complicated the analysis of the immunofluorescence data, for staining with MS110 antibody the infection was carried out at an m.o.i of 3 instead of the usual 20. The cells stained with MS110 exhibited different pattern of staining compared to that observed with the previous antibodies. The non-infected cells showed a faint and principally cytoplasmic staining (Fig. R22). This result was in apparent contrast with data from other groups that showed a predominantly nuclear staining pattern, specifically a punctate nuclear signal, using this antibody (Scully, Ganesan et al. 1996; Hsu and White 1998; Wilson, Ramos et al. 1999; Yoshikawa, Honda et al. 1999; Tulchin, Chambon et al. 2010). HSV-1 infection led to an increased staining with MS110 that was homogeneous in both cytoplasm and nucleus.

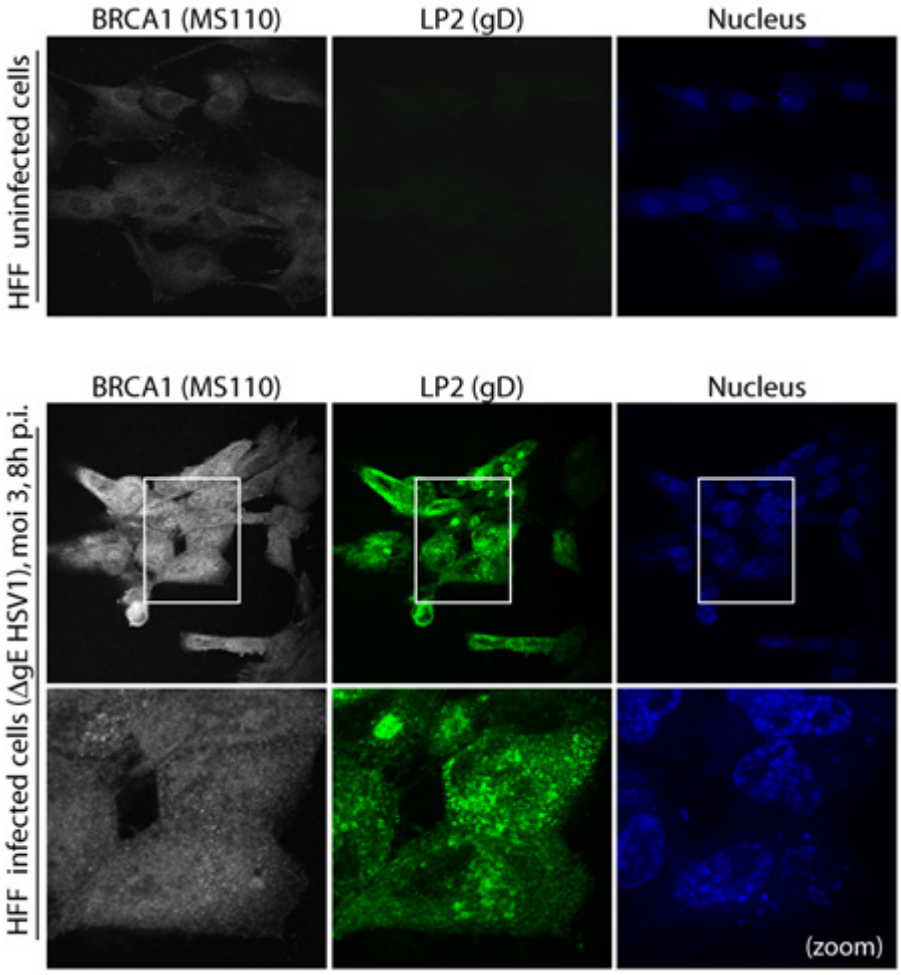


Figure R22. Staining with the MS110 antibody shows faint cytoplasmic localization in noninfected cells and increased signal in both cytoplasm and nucleus during HSV-1 infection of HFF cells. HFF cells were mock or infected with ΔgE (3 p.f.u/cell). 8 h.p.i uninfected and infected cells were fixed in 2% paraformaldehyde and stained with anti-BRCA1 MS110 plus goat anti-mouse IgG1 Alexa Fluor 633 (grey, left panel) and anti-gD LP2 plus goat anti-mouse IgG2a Alexa Fluor 488 (green, middle panel) as a control of infection. Nuclei were stained with DAPI (blue, right panel).

Taken together, the data obtained with the three BRCA1 antibodies did not exhibit the characteristic nuclear localization of BRCA1, predominantly in nuclear speckles, previously reported (Scully, Ganesan et al. 1996; Hsu and White 1998; Wilson, Ramos et al. 1999; Yoshikawa, Honda et al. 1999; Tulchin, Chambon et al. 2010) and they did not show a consistent change in the localization of BRCA1 staining between uninfected and HSV-1 infected cells as we had hypothesized.

To determine whether the data obtained on analysis of the HFF cells were consistent in other cells susceptible to HSV-1 infection, the highly permissive VERO cell line was mock infected or infected with HSV-1 at m.o.i.s of 3 or 1, as indicated, and subjected to immunofluorescence analysis 8 h.p.i using I-20, D-20 and MS110 antibodies.

The pattern of staining observed in Vero cells using I-20 (Fig. R23) was very similar to that observed in HFF cells (Fig. R20). However, the signal in the HSV-1 infected Vero cells was much more intense, so much so that the exposure had to be reduced to be able to discern properly the pattern of staining.

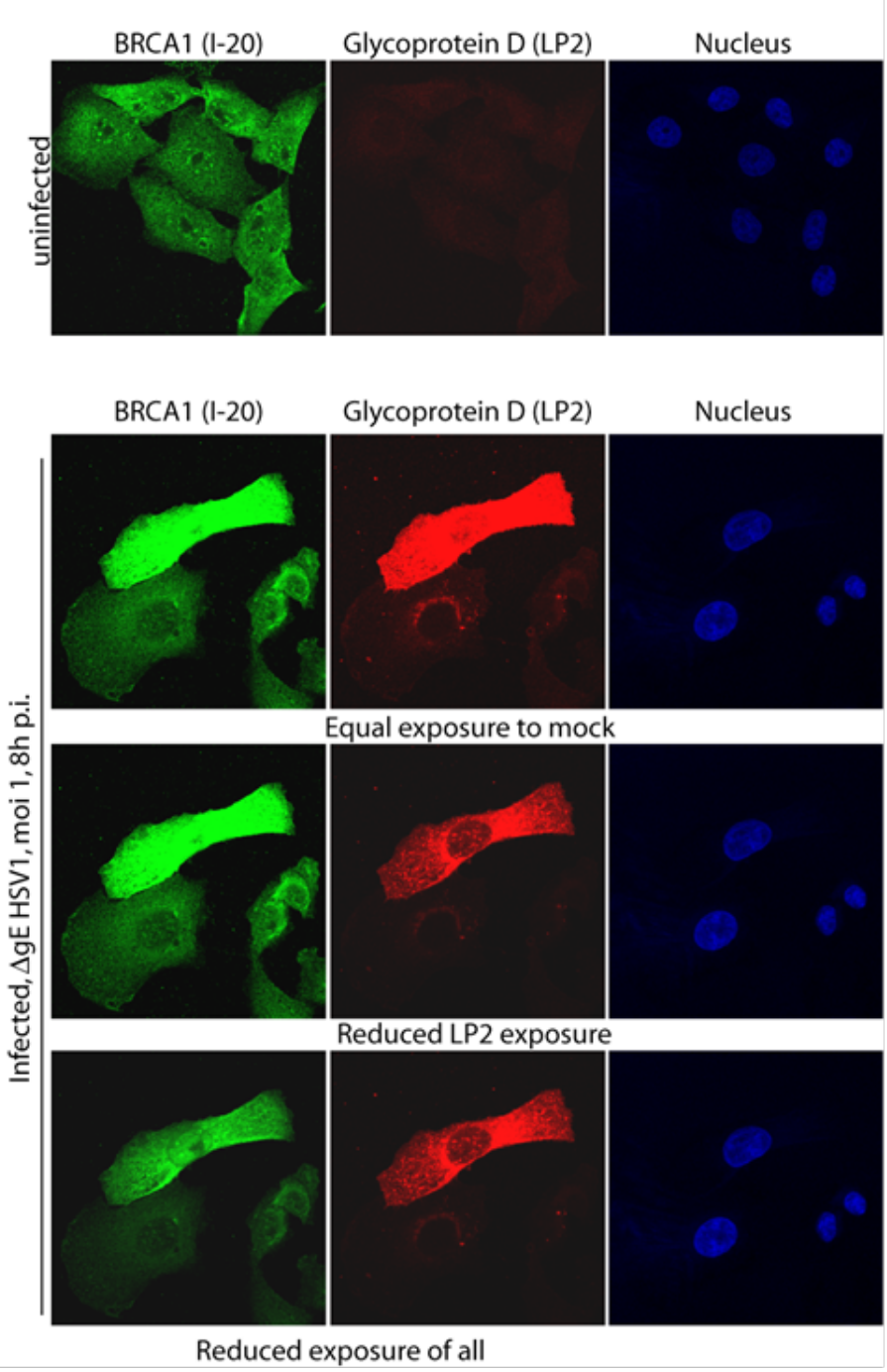


Figure R23. The staining with I-20 shows an increased signal, in both nucleus and cytosol, during HSV-1 infection of VERO cells. VERO cells were mock or infected with ΔgE (1 p.f.u/cell). 8 h.p.i uninfected and infected cells were fixed in 2% paraformaldehyde and stained with anti-BRCA1 I-20 plus goat anti-rabbit Alexa Fluor 488 (green, left panel) and anti-gD LP2 plus donkey anti-mouse Alexa Fluor 568 (red, middle panel) as a control of infection. Nuclei were stained with DAPI (blue, right panel).

Staining with D-20 showed no differences in intensity or localization between uninfected and infected Vero cells (Fig. R24), being consistent with that observed in HFF cells (Fig. R21).

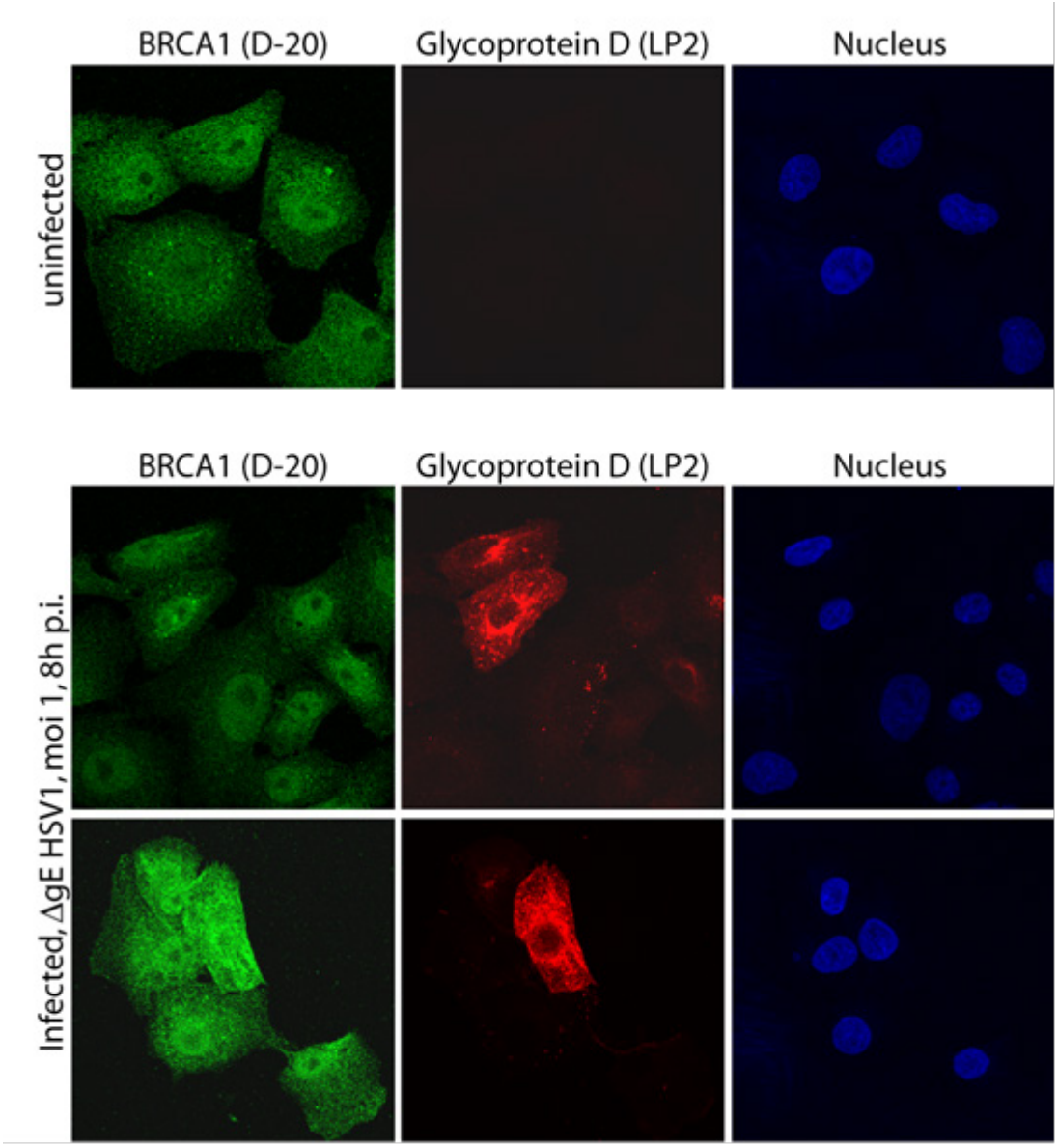


Figure R24. The staining with D-20 shows no differences between non-infected and infected VERO cells. VERO cells were mock or infected with ΔgE (1 p.f.u/cell). 8 h.p.i uninfected and infected cells were fixed in 2% paraformaldehyde and stained with anti-BRCA1 D-20 plus goat anti-rabbit Alexa Fluor 488 (green, left panel) and anti-gD LP2 plus donkey anti-mouse Alexa Fluor 568 (red, middle panel) as a control of infection. Nuclei were stained with DAPI (blue, right panel).

The uninfected Vero cells showed no staining using MS110. As stated above, this result was in contrast with previous reports that showed a predominantly nuclear staining using this antibody. During HSV-1 infection cells displayed a predominantly nuclear staining with less marked cytoplasmic signal (Fig. R25). This pattern of labeling was completely different from that observed in HFF cells, although in both cell lines HSV-1 infection led to an increased staining (Fig. R22). Indeed, during infection of Vero cells the staining was more intense at higher m.o.i.. Moreover, at m.o.i. 1 the signal was mainly nuclear but at m.o.i. 3 the cytoplasmic staining was considerably increased.

Taken together, the data obtained in HFF and Vero cells using the BRCA1 antibodies I-20, D-20 and MS110 did not exhibit the characteristic nuclear dot staining pattern of BRCA1 previously reported. Moreover, these experiments did not show a change in the localization of BRCA1 staining during HSV-1 infection as we had hypothesized that might happen if BRCA1 was cleaved (Fig. R19).

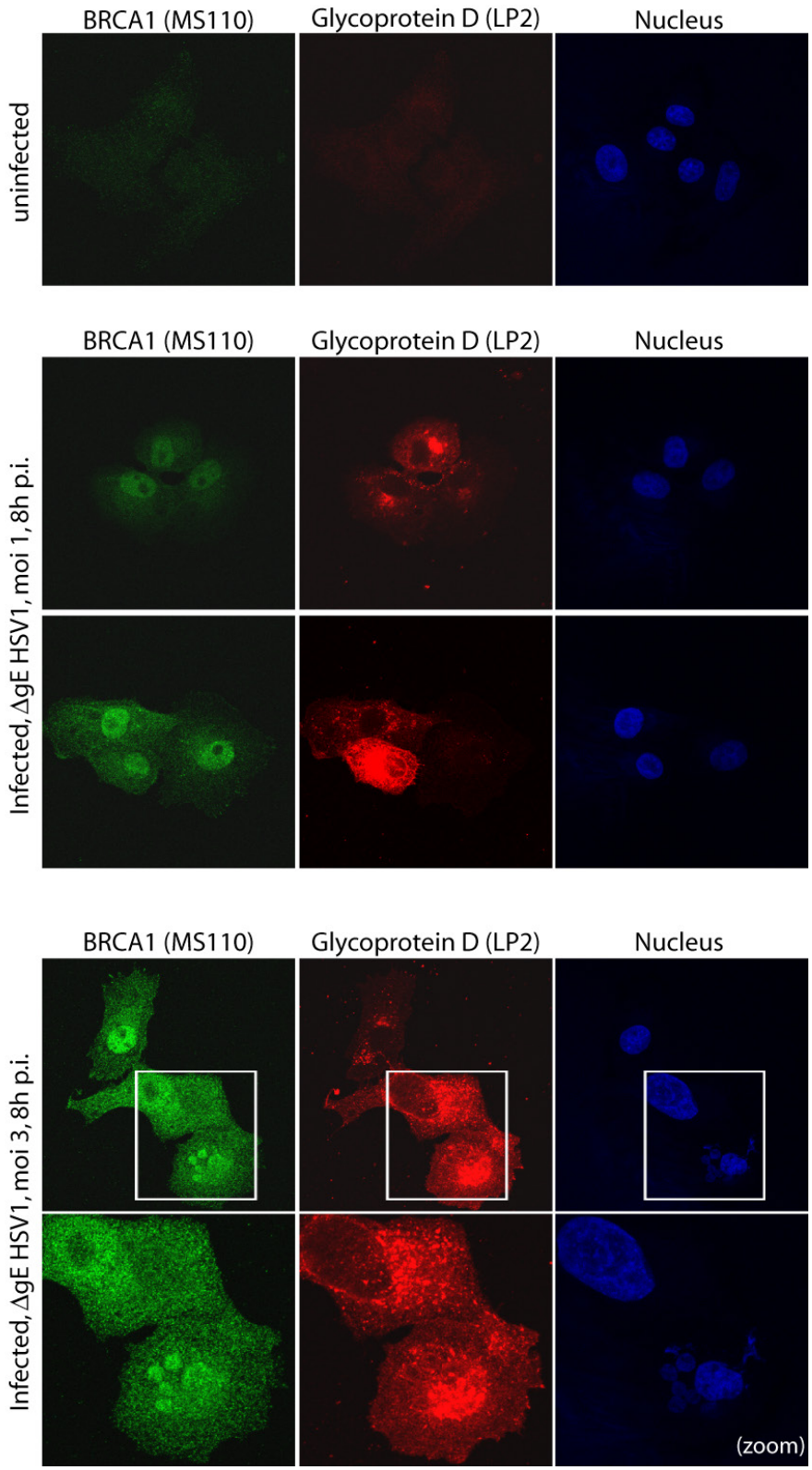


Figure R25. MS110 staining shows no labeling in non-infected cells and a predominantly nuclear staining with faint cytoplasmic signal during HSV-1 infection of VERO cells. VERO cells were mock or infected with ΔgE (1 p.f.u/cell). 8 h.p.i uninfected and infected cells were fixed in 2% paraformaldehyde and stained with anti-BRCA1 MS110 plus goat anti-mouse IgG1 Alexa Fluor 488 (green, left panel) and anti-gD LP2 plus goat anti-mouse IgG2a Alexa Fluor 568 (red, middle panel) as a control of infection. Nuclei were stained with DAPI (blue, right panel).

Since the predominantly nuclear localization of full-length BRCA1 previously described was not observed in our immunofluorescence analyses of HFF and Vero cells, and levels of this 220 KDa protein appeared non-detectable in our immunoblot analyses, a positive-control system using cells transfected to express full-length BRCA1 was employed to test the hypothesis of the BRCA1 cleavage during HSV-1 infection. Specifically, a U2OS cell line that stably expressed full-length BRCA1 fused in its N-terminal region to GFP (J. Lukas, personal communication) (GFP-BRCA1 Flag-BARD1), kindly provided by Steve Jackson's lab (The Gurdon Institute, University of Cambridge) (Mailand, Bekker-Jensen et al. 2007; Galanty, Belotserkovskaya et al. 2009), was used. These cells also express BARD1 to avoid the mislocalization of BRCA1 as explained before. This is of particular importance because deregulation of nuclear transport pathways can cause not only the mislocalization of the proteins, but also the alteration of their functions (reviewed in (Hung and Link 2011)).
The amount of bright GFP positive cells was low (around 20% of positive cells as measured by fluorescence microscopy using anti-GFP and anti-FLAG antibodies) complicating the analyses. Thus initially, while we tried to enrich the positive population by single-cell cloning, an anti-GFP antibody was used to enhance the signal in immunofluorescence analysis.
To evaluate how HSV-1 infection affected the subcellular localization of full-length BRCA1, the stably transfected U2OS cells were either mock infected or infected with HSV-1 ΔgE and immunofluorescence analysis was done using antibodies to GFP and gD as a control of infection (Fig. R26).
Using this system, the characteristic punctate nuclear staining of full-length BRCA1 was seen in the uninfected cells, but after HSV-1 infection most BRCA1 accumulations were dispersed throughout the nucleus (Fig. R26). This result was consistent with previous reports, which indicated that wt HSV-1 infection dispersed BRCA1 from its nuclear accumulations and redistributed BRCA1 diffusely in the nucleus (Maul, Jensen et al. 1998; Lilley, Chaurushiya et al. 2011). No shift in the subcellular localization of BRCA1 was observed after HSV-1 infection, but this could be explained due to the detection of GFP fused to the N-terminus of full-length BRCA1, which is the region predicted to remain in the nucleus, if the molecule is proteolysed (Fig. R19).

The availability of this cell line expressing GFP-BRCA1 allowed a better characterization of the signal recognized by the BRCA1 antibodies I-20, MS110 and D-20 in immunofluorescence analysis.
Immunofluorescence using the I-20 antibody showed a predominantly nuclear staining in cells that overexpressed GFP-BRCA1 full-length, which overlapped with the GFP signal. In cells with lower levels of GFP-BRCA1 expression, as judged by the GFP staining, the I-20 antibody yielded an apparent nuclear dot staining (Fig. R27). Therefore, in cells overexpressing full-length BRCA1, I-20 staining showed the expected nuclear pattern, indicating that this antibody is able to recognize transfected full-length BRCA1.

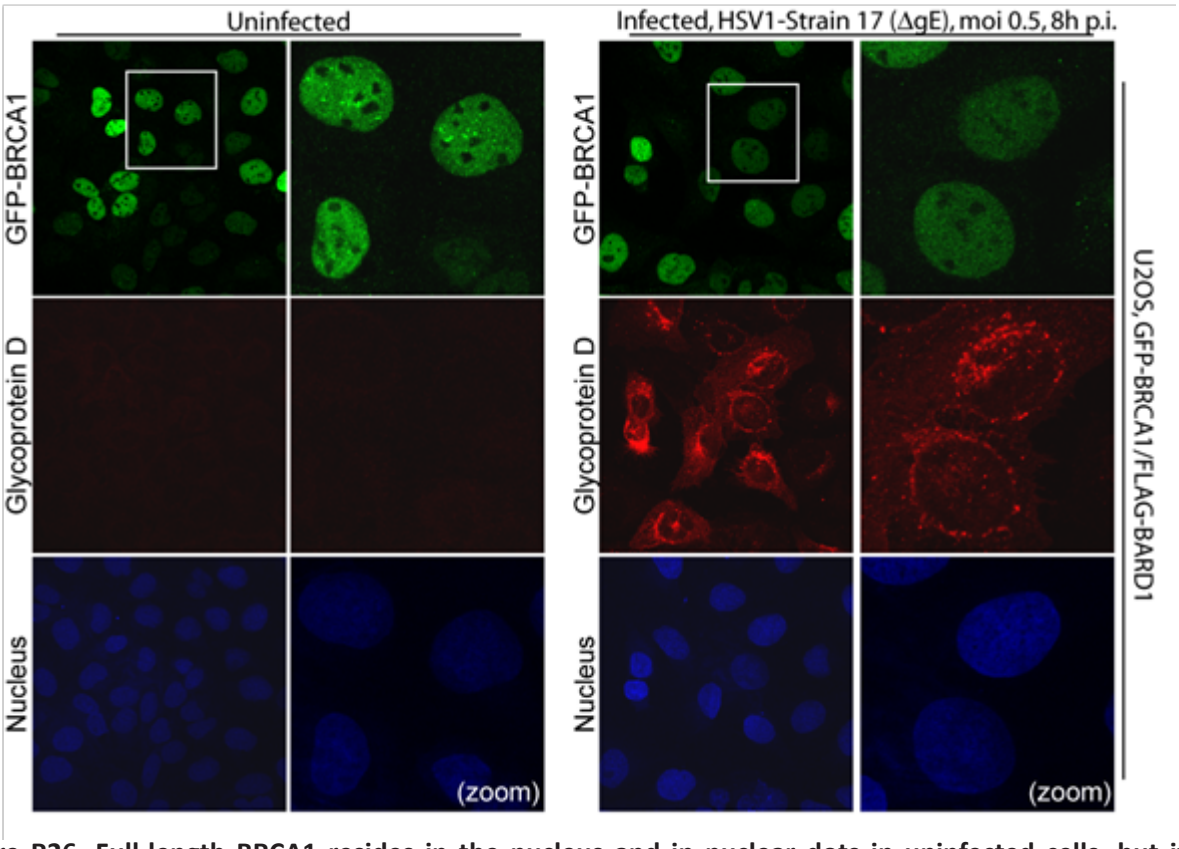


Figure R26. Full-length BRCA1 resides in the nucleus and in nuclear dots in uninfected cells, but it is redistributed diffusely in the nucleus during HSV-1 infection. U2OS stably expressing BRCA1 cells were mock or infected with ΔgE (0,5 p.f.u/cell). 8 h.p.i uninfected and infected cells were fixed in 2% paraformaldehyde and stained with anti-GFP plus goat anti-rabbit Alexa Fluor 488 (green, left panel) and anti-gD LP2 plus donkey anti-mouse Alexa Fluor 568 (red, middle panel) as a control of infection. Nuclei were stained with DAPI (blue, right panel).

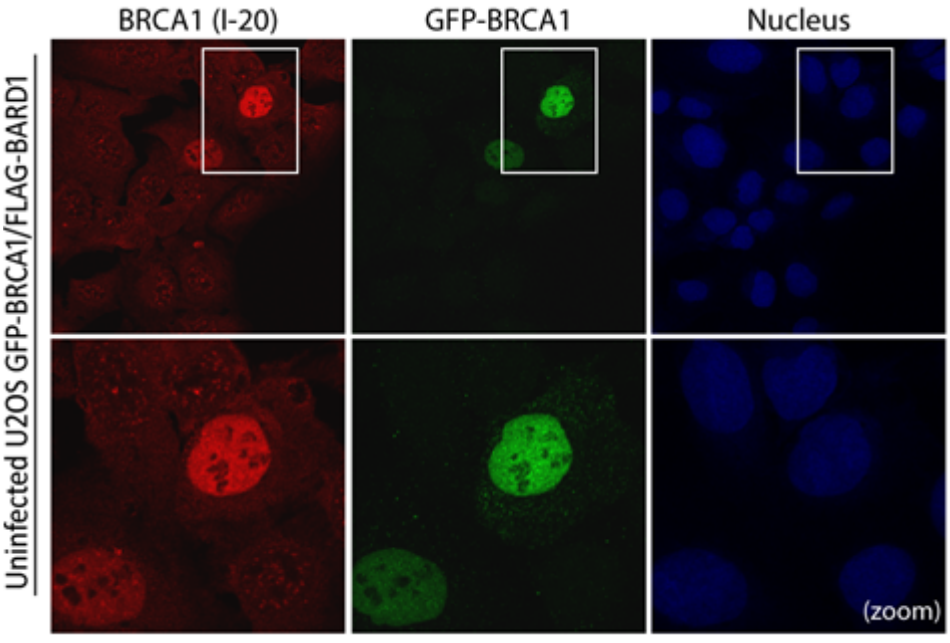


Figure R27. I-20 recognized full-length BRCA1 in the nucleus. U2OS stably expressing BRCA1 cells were fixed in 2% paraformaldehyde and stained with anti-BRCA1 I-20 plus goat anti-rabbit Alexa Fluor 568 (red, left panel) and anti-GFP (clone JL-8) plus goat anti-mouse Alexa Fluor 488 (green, middle panel). Nuclei were stained with DAPI (blue, right panel).

Once the ability of the I-20 antibody to bind full-length BRCA1 was confirmed, the effects of HSV-1 infection on the pattern of staining were studied. These experiments were performed using a new batch of cells with stronger GFP-BRCA1 signal obtained by single cell cloning of the stable U2OS cell line. This enabled us to do the immunofluorescence analysis without the anti-GFP antibody. These cells were mock infected or infected with ΔgE HSV-1, and the infection was checked using the LP2 anti-gD antibody (Fig. R28).

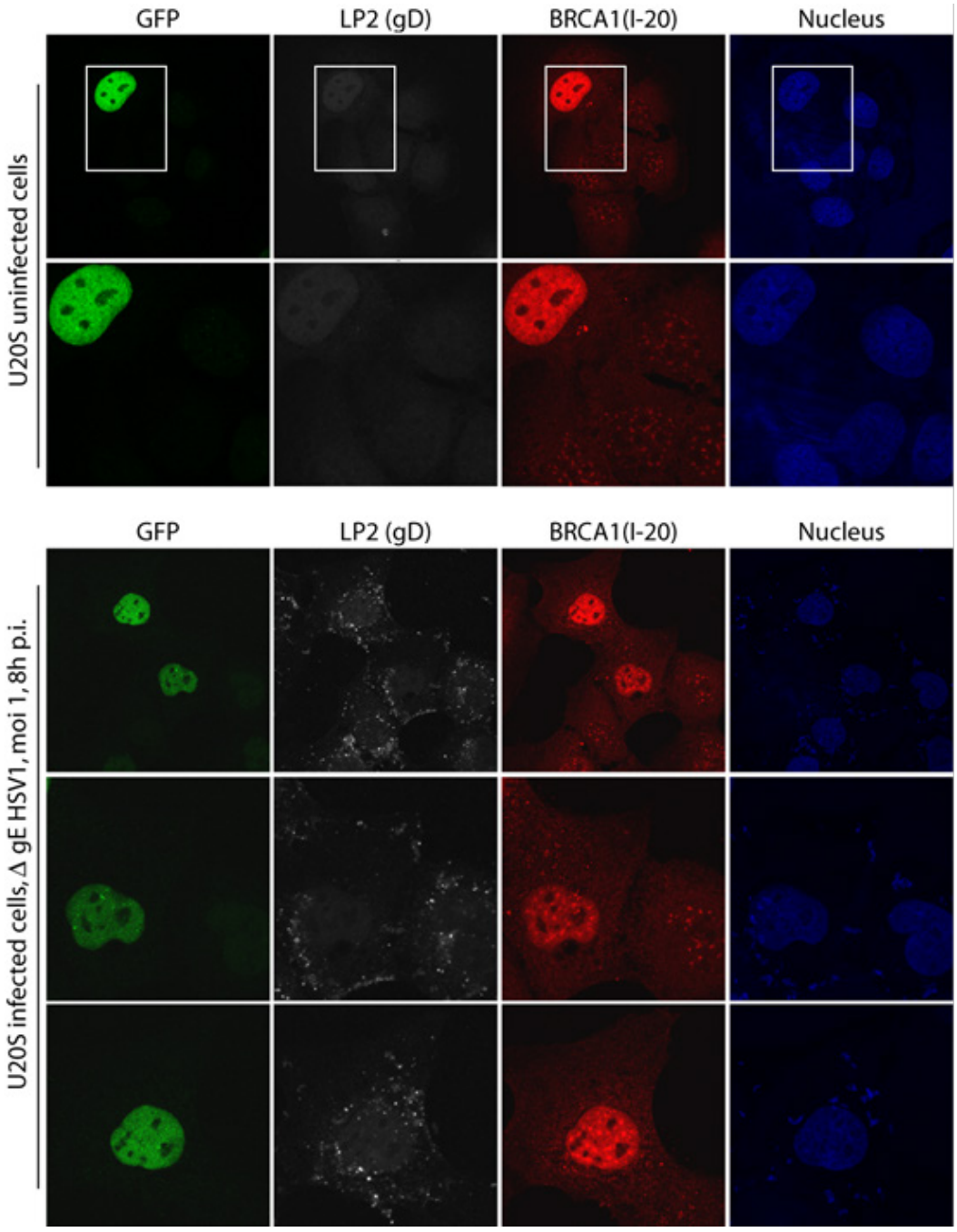


Figure R28. I-20 staining does not reveal any change in subcellular localization signal during HSV-1 infection of U2OS stably expressing full-length BRCA1. U2OS stably expressing BRCA1 cells were mock or infected with ΔgE (1 p.f.u/cell). 8 h.p.i uninfected and infected cells were fixed in 2% paraformaldehyde and stained with I-20 plus goat anti-rabbit Alexa Fluor 568 (red) and anti-gD LP2 plus goat anti-mouse Alexa Fluor 633 (grey) as a control of infection. GFP fluorescence was visualized directly (green). Nuclei were stained with DAPI (blue). Magnification 40X.

These experiments showed that no significant changes in GFP-BRCA1 localization were detected during HSV-1 infection of U2OS stably expressing full-length BRCA1 using the I-20 antibody (Fig. R28).

This experiment was then repeated with single cell cloned cells expressing full-length BRCA1, isolated using Fluorescence Activated Cell Sorting (FACS) and replated to generate a monoclonal lineage (Fig. R29). Although we could see stronger GFP-BRCA1, the staining with I-20 again failed to show significant changes in localization during HSV-1 infection.

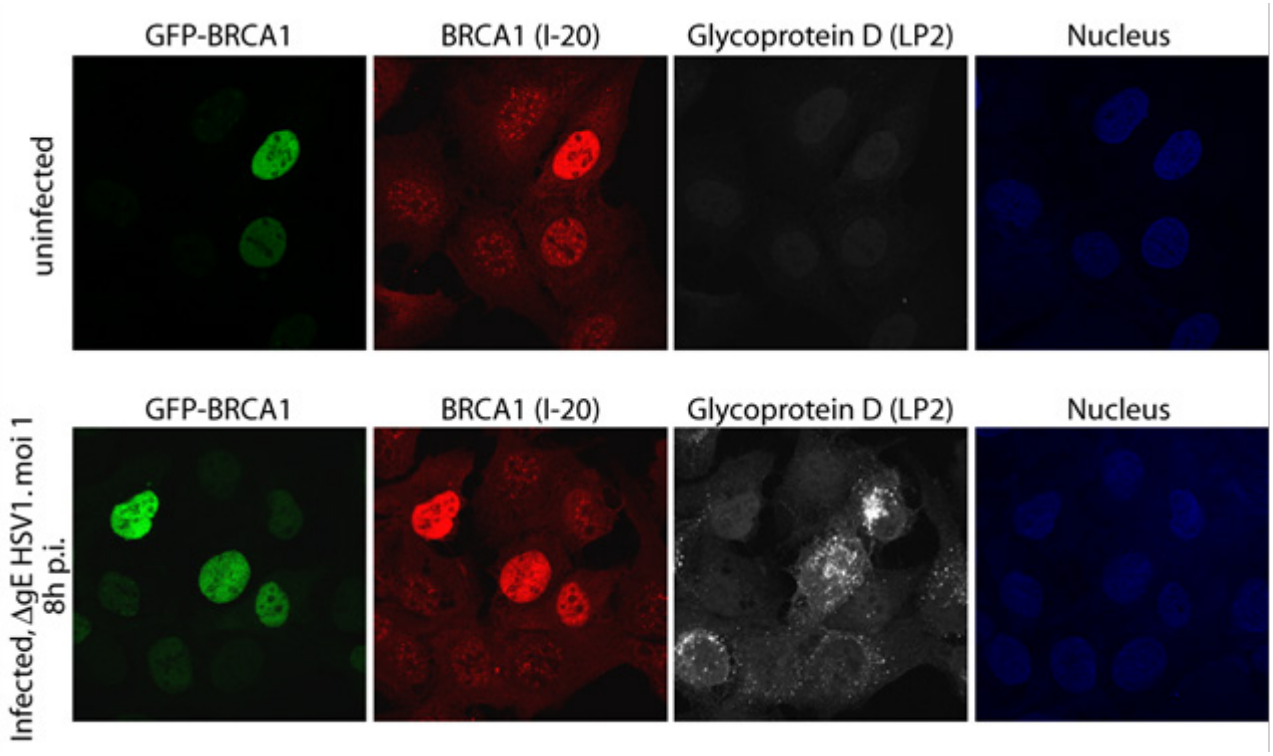


Figure R29. I-20 staining does not reveal any change in subcellular localization signal during HSV-1 infection of sorted U2OS stably expressing full-length BRCA1. U2OS stably expressing BRCA1 cells were mock or infected with ΔgE (1 p.f.u/cell). 8 h.p.i uninfected and infected cells were fixed in 2% paraformaldehyde and stained with I-20 plus goat anti-rabbit Alexa Fluor 568 (red) and anti-gD LP2 plus goat anti-mouse Alexa Fluor 633 (grey) as a control of infection. GFP fluorescence was visualized directly (green). Nuclei were stained with DAPI (blue).

The staining of these sorted cells with MS110 (Fig. R30) gave a nuclear pattern which was consistent with the GFP signal. This result indicated that MS110 antibody recognized transfected full-length BRCA1. HSV-1 infection did not lead to significant changes in the localization of full-length BRCA1, however, if the molecule is fractionated, the N-terminal region recognized by this antibody would be expected to be retained in the nucleus.

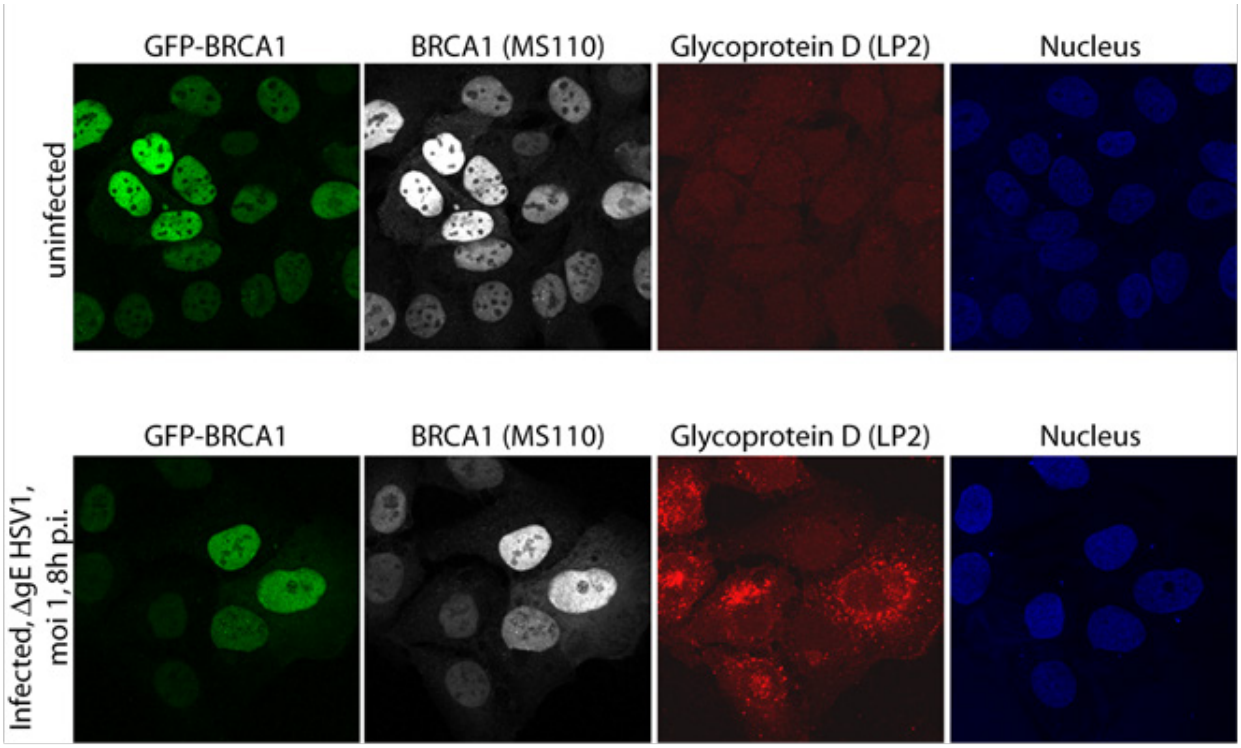


Figure R30. MS110 recognized BRCA1 in the nucleus but MS110 staining does not reveal any change in subcellular localization signal during HSV-1 infection of sorted U2OS stably expressing full-length BRCA1. U2OS stably expressing BRCA1 cells were mock or infected with ΔgE (1 p.f.u/cell). 8 h.p.i uninfected and infected cells were fixed in 2% paraformaldehyde and stained with MS110 plus goat anti-mouse IgG1 Alexa Fluor 633 (grey) and anti-gD LP2 plus goat anti-mouse IgG2a Alexa Fluor 568 (red) as a control of infection. GFP fluorescence was visualized directly (green). Nuclei were stained with DAPI (blue).

Since the D-20 antibody did not recognize full-length BRCA1 in the stably transfected U2OS cells (Fig. 34) we did not study further the pattern of staining during HSV-1 infection with this antibody. This result pointed out the unreliability of this antibody.

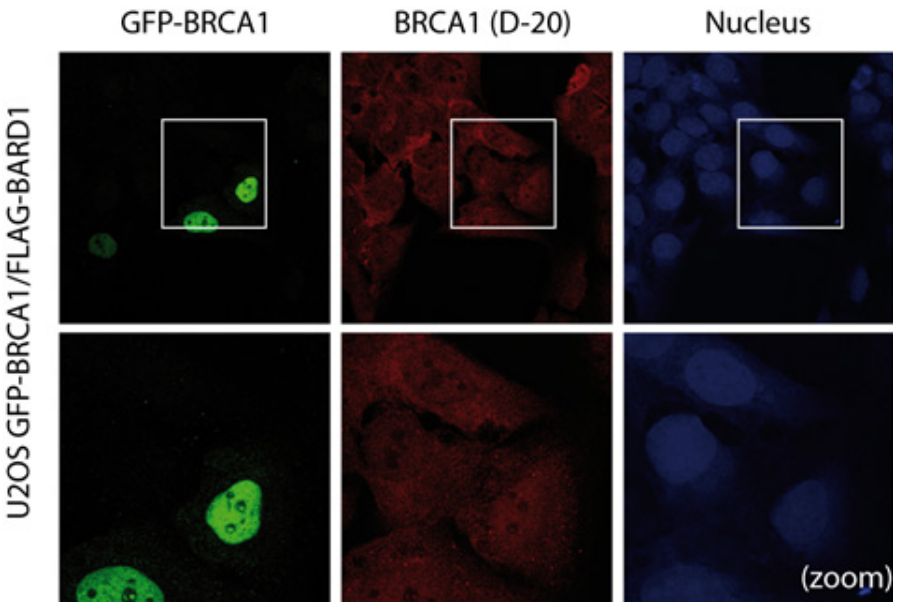


Figure R31. D-20 does not recognize full-length BRCA1. U2OS stably expressing BRCA1 cells were fixed in 2% paraformaldehyde and stained with anti-BRCA1 D-20 plus goat anti-rabbit Alexa Fluor 568 (red, left panel) and anti-GFP (clone JL-8) plus goat anti-mouse Alexa Fluor 488 (green, middle panel). Nuclei were stained with DAPI (blue, right panel).

These data verified that **I-20 and MS110**, **but not D-20**, **recognized full-length transfected BRCA1** within the nucleus in immunofluorescence analysis, but the staining with these antibodies did not reveal significant changes in localization during HSV-1 infection as we had hypothesized (Fig. R19).

Since no significant changes in BRCA1 localization were observed by immunofluorescence analysis, immunoprecipitation-immunoblot analyses of the cells that stably expressed BRCA1 at high levels were performed simultaneously. GFP molecular weight is 27KDa, so, if BRCA1 full-length was cleaved during HSV-1 infection originating the 120KDa fragment observed with the I-20 antibody, a band around 100 KDa (80+27) would be also expected using an antibody against GFP or antibodies which recognized an epitope in the N-terminus part of the molecule (D-20, MS110) (Fig. R32).

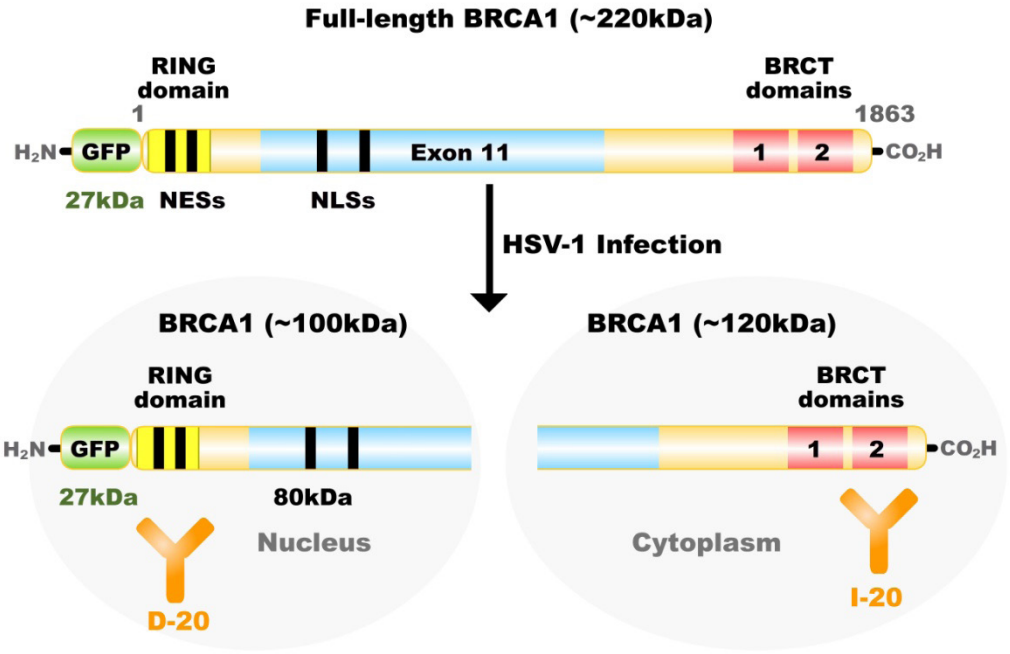


Figure R32. Schematic representation of the hypothetical cleavage of GFP-BRCA1 mediated by HSV-1. A cleavage of GFP-BRCA1 protein mediated by HSV-1 infection would originate a fragment of 120 kDa, recognized by the I-20 antibody, respectively. originating the 120KDa observed with the I-20 antibody, a band around 100 KDa (80+27) would be also expected using an antibody against GFP or antibodies which recognized an epitope in the N-terminus part of the molecule (D-20, MS110)

For this experiment, the stably transfected U2OS cells were either mock infected or infected with the ΔgE virus at m.o.i of 5. 24 h.p.i cell lysates from uninfected and infected cells were immunoprecipitated with a rabbit polyclonal antibody anti-GFP and then subjected to immunoblotting using a mouse monoclonal antibody anti-GFP. Figure R33 shows that a specific band could be detected running above the 230-KDa marker, consistent with the expression by these cells of the tagged full-length BRCA1, higher than 220-230 KDa due to the fusion to the GFP tag. However, the smaller band of around 100 KDa expected in the lane of the infected cells was not observed. The full-length BRCA1 band was not seen in the total lysates, but this might reflect that the lysates were too dilute.

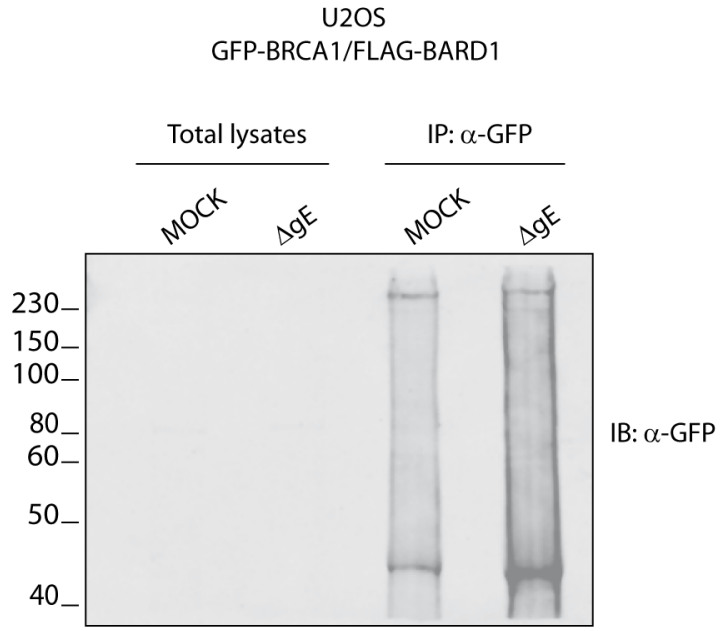


Figure R33. BRCA1 from U2OS stably transfected with full-length GFP-BRCA1 construct was detected by immunoprecipitation and immunoblot analysis using anti-GFP antibodies. U2OS stably expressing BRCA1 cells were mock infected or infected with ΔgE virus (5 pfu/cell). 24 h.p.i cell lysates were immunoprecipitated with a rabbit polyclonal antibody anti-GFP and immunoblotted with a mouse monoclonal antibody anti-GFP (clone JL-8).

Also, lysates of the positive control cells, uninfected and infected, were immunoprecipitated with anti-GFP antibodies followed by immunoblotting with I-20 and D-20, as well as GFP antibodies, to check if I-20 and D-20 were able to recognise immunoprecipitated full-length BRCA1 and to test if any smaller bands appeared in the lane of the infected cells using these antibodies (Fig. R34). MS110 was not used in the immunoprecipitation/immunoblotting analysis because in our previous experiments MS110 did not work in western blot. Moreover, despite its success detecting BRCA1 in immunohistochemistry analysis, previous findings indicated its lack of specificity in western blot analysis (Fraser, Reeves et al. 2003; Milner, Wombwell et al. 2013).
These experiments were performed using FACS sorted cells for higher GFP-BRCA1 expression and they were infected with a reduced m.o.i of 1 pfu/cell that still achieved a good infection.

As shown in Figure R34 the I-20 and D-20 antibodies recognised immunoprecipitated full-length BRCA1, but no differences were observed between mock and infected cells. In particular, the smaller band of 100 KDa expected after HSV-1 infection using anti-GFP and D-20 antibodies (which would recognized the N-terminal fraction of BRCA1) was not detected. Moreover, the band around 80kDa previously detected by the D-20 antibody in total lysates of infected cells (Figs. R15, R16, R17) was not clearly observed.

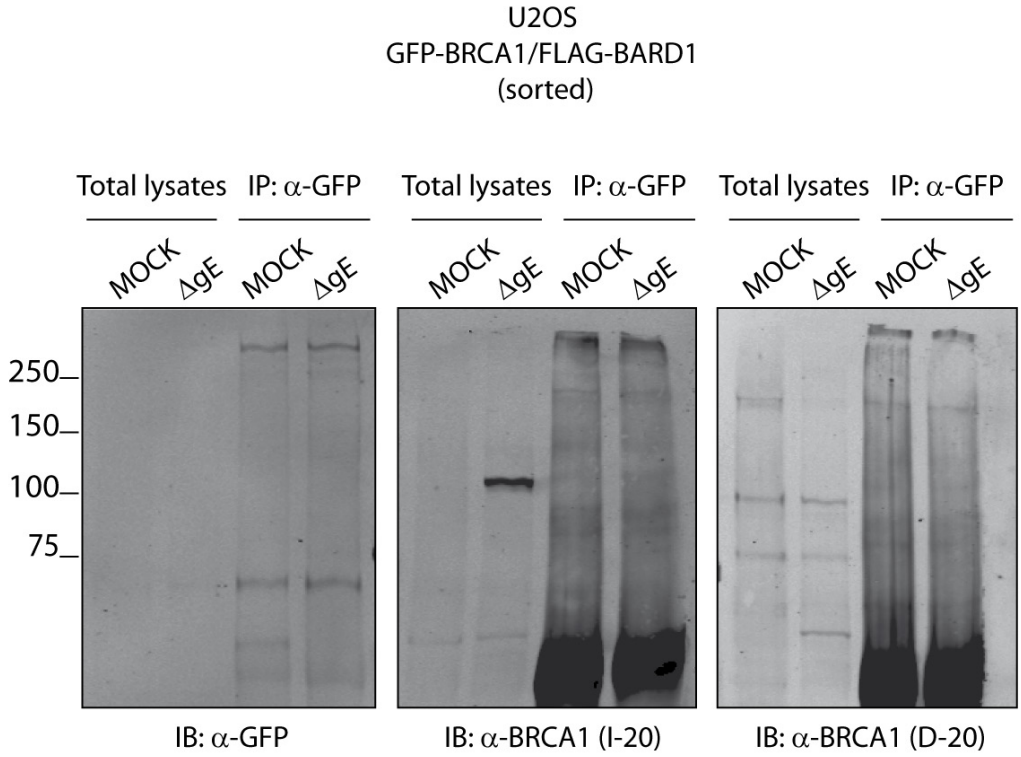


Figure R34. I-20 and D-20 detect overexpressed full-length BRCA1 in FACS sorted U2OS cells stably expressing GFP-BRCA1 by immunoprecipitation with anti-GFP and immunoblot analysis using I-20 and D-20. U2OS stably expressing BRCA1 were mock infected or infected with ΔgE virus (1 pfu/cell). 24 h.p.i cell lysates were immunoprecipitated with a polyclonal antibody anti-GFP and immunoblotted with the monoclonal antibody anti-GFP (clone JL-8), the I-20 antibody or the D-20 antibody.

The fact that the 120 kDa band was not observed by immunoblot analysis with I-20 of anti-GFP immunoprecipitates from HSV-1 infected cells (Fig. R34) could be consistent with our cleavage hypothesis. As the region precipitated by GFP is the N-terminal, if a cleavage of BRCA1 took place, the C-terminal region recognized by the I-20 antibody would not be precipitated. If this was the case, immunoprecipitation of the unbound fraction from the previous immunoprecipitation (Fig. R34) using the I-20 antibody should show the 120 KDa-protein. This experiment confirmed this prediction (Fig. R35). However, while it is possible that this band represent a 120 KDa fragment produced by BRCA1 cleavage, the fact that full-length BRCA1 could not be detected by the I-20 antibody in total lysates leaves open the possibility that this 120 KDa band could be another target (inespecificity).

Overall, these data confirm that **I-20 and D-20 antibodies recognize the transfected GFP-BRCA1 fusion protein** in anti-GFP immunoprecipitates, but the smaller band around 100KDa expected with the N-terminal antibodies was not seen. Moreover these immunoprecipitation-immunoblot analyses correlated with the immunofluorescence stainings of the U2OS stably expressing full-length BRCA1, suggesting that the full-length molecule was not cleaved during HSV-1 infection. However, it could not be excluded that the cleavage might be prevented by the presence of the GFP fusion. For

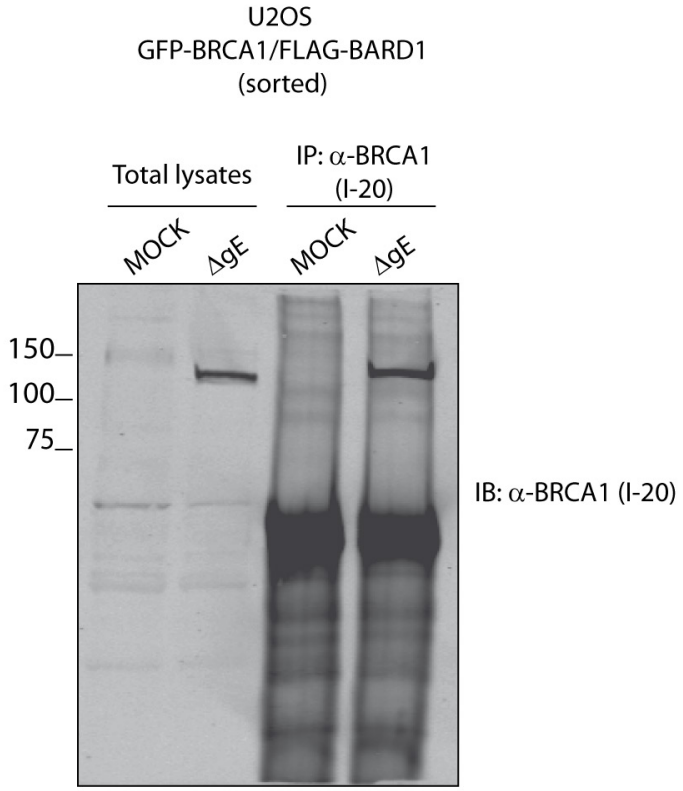


Figure R35. I-20 immunoprecipates the 120 KDa-protein in the unbound fraction from an immunoprecipitation using anti-GFP. The unbound fraction from the previous immunoprecipitation was immunoprecipitated with I-20 antibody and immunoblotted with the same antibody. Total cell lysates were also immunoblotted with I-20.

example, due to the GFP tagged to the BRCA1, a possible blocking accessibility for cellular or viral proteins to correctly bind and adequately cleave BRCA1 could explain these results.

All the previous data, both immunofluorescence and immunoprecipitation analyses, indicated that the I-20 antibody efficiently recognized full-length BRCA1 protein fused to GFP, but they suggested that full-length BRCA1 was not cleaved during HSV-1 infection, as although the 120 KDa protein can be found in total lysates of infected GFP-BRCA1 cells, the 100 KDa protein was not detected. Thus, the identity of the 120KDa protein recognized by the I-20 antibody was still an incognita.

2.6. Identification of the 120 KDa-protein by proteomic analysis.

Given that the immunofluorescence staining with I-20 was so intense in Vero cells (Fig. R23), presumably reflecting a higher level of expression of the I-20 reactive material, we decided to carry out an immunoprecipitation with I-20 from mock and HSV-1 infected vero cells lysates to try to obtain sufficient material to allow a proteomic identification of the 120 KDa protein (Fig. R36).

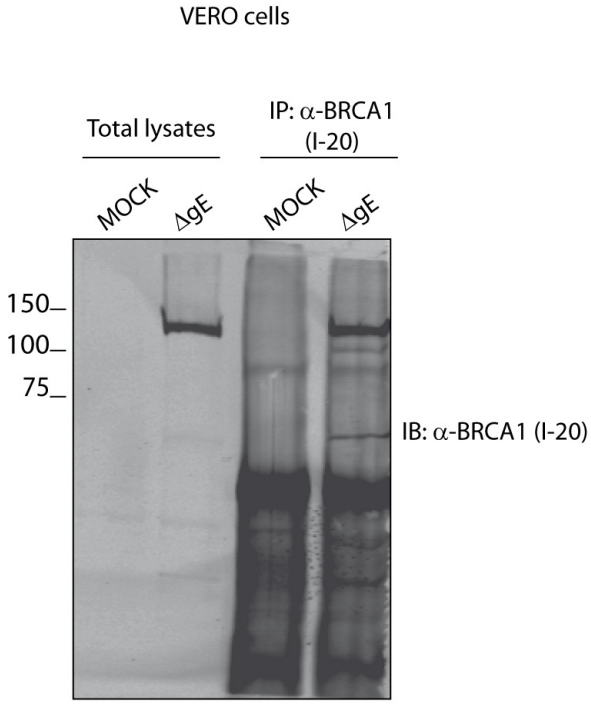


Figure R36. I-20 immunoprecipates the 120 KDa-protein in HSV-1 infected VERO cells. VERO cells were mock infected or infected with ΔgE virus (1 pfu/cell). 24 h.p.i cell lysates were immunoprecipitated with I-20 antibody and immunoblotted with the same antibody. Total cell lysates were also immunoblotted with I-20.

Given that the 120 KDa-protein was successfully immunoprecipitated (Fig. R36), the immunoprecipitated proteins were again separated by SDS-PAGE and the resulting gel was stained with Coomassie dye. The 120 KDa band was visualized in the Coomassie-stained gel together with other bands in the infected cells, especially one around 150 KDa as well as the one at 80 KDa which had been observed in some of our previous western blot analysis using the I-20 antibody (Fig. R37). These proteins (150, 120 and 80 KDa) were then processed for MALDI Fingerprinting identification.

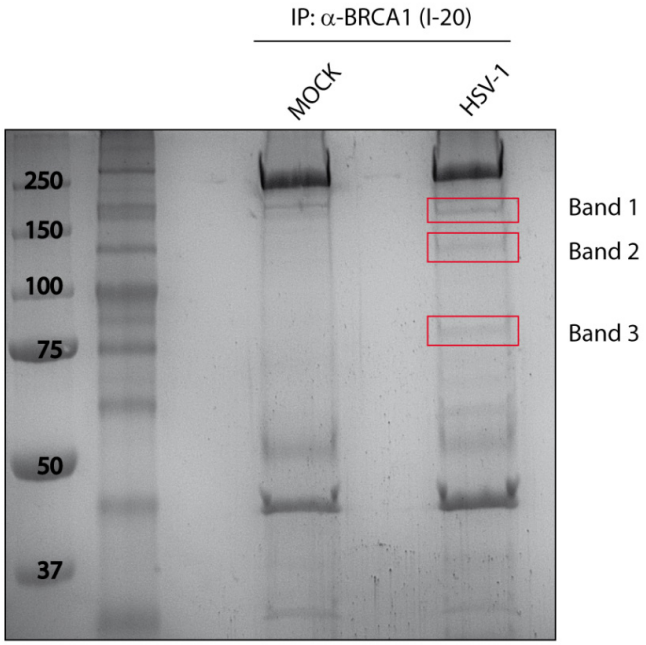


Figure R37. Coomassie stained SDS-PAGE gel showing the three immnunoreactive bands which were subjected to mass spectrometry analysis. Bands of interest are indicated by red boxes.

The mass spectrometry analysis identified the **120 KDa-protein** (band number 2) as the **viral tegument protein UL37** from human herpesvirus 1 (also called Capsid assembly protein UL37) with 55 peptides corresponding to 57.8 % sequence coverage (Fig. R38). The predicted molecular weight of UL37 is 120 KDa (http://www.ncbi.nlm.nih.gov/protein/222478368/).
The other two proteins were also identified as viral proteins:

- **Band 1**: major capsid protein (human herpesvirus 1) (also called Capsid protein VP5), which has a predicted molecular weight of 149 kDa.
- **Band 3**: tegument protein VP13/14 (human herpesvirus 1) (also called tegument protein UL47), which has a predicted molecular weight of 74 kDa.

The antigen used to generate the anti-BRCA1 antibody I-20 is the sequence between 1823-1842 aminoacids located within the BRCT2 domain: AIGQMCEAPVVTREWVLDSV. Alignment between the peptide used to generate the anti-BRCA1 antibody I-20 and HSV-1 UL37 protein revealed a similarity of six consecutive identical amino acids at the N-terminus region of UL37 (Fig. R38).

A

UL37 (gi|222478368)
Length: 1123 aa
Mass: 120.556 kDa
Sequence coverage: 57.8 %
Sequence:

```
   1 MADRGLPSEA PVVTTSPAGP PSDGPMQRLL ASLAGLRQPP TPTAETANGA
  51 DDPAFLATAK LRAAMAAFLL SGTAIAPADA RACWRPLLEH LCALHRAHGL
 101 PETALLAENL PGLLVHRLVV ALPEAPDQAF REMEVIKDTI LAVTGSDTSH
 151 ALDSAGLRTA AALGPVRVRQ CAVEWIDRWQ TVTKSCLAMS PRTSIEALGE
 201 TSLKMAPVPL GQPSANLTTP AYSLLFPAPF VQEGLRFLAL VSNRVTLFSA
 251 HLQRIDDATL TPLTRALFTL ALVDEYLTTP ERGAVVPPPL LAQFQHTVRE
 301 IDPAIMIPPL EANKMVRSRE EVRVSTALSR VSPRSACAPP GTLMARVRTD
 351 VAVFDPDVPF LSSSALAVFQ PAVSSLLQLG EQPSAGAQQR LLALLQQTWI
 401 LIQNTNSPSV VINTLIDAGF TPSHCTHYLS ALEGFLAAGV PARTPTGHGL
 451 GEVQQLFGCI ALAGSNVFGL AREYGYYANY VKTFRRVQGA SEHTHGRLCE
 501 AVGLSGGVLS QTLARIMGPA VPTEHLASLR RALVGEFETA ERRFSSGQPS
 551 LLRETALIWI DVYGQTHWDI TPTTPATPLS ALLPVGQPSH APSVHLAAAT
 601 QIRFPALEGI HPNVLADPGF VPYVLALVVG DALRATCSAA YLPRPVEFAL
 651 RVLAWARDFG LGYLPTVEGH RTKLGALITL LEPAARGGLG PTMQMADNIE
 701 QLLRELYVIS RGAVEQLRPL VQLQPPPPPE VGTSLLLISM YALAARGVLQ
 751 DLAERADPLI RQLEDAIVLL RLHMRTLSAF FECRFESDGR RLYAVVGDTP
 801 DRLGPWPPEA MGDAVSQYCS MYHDAKRALV ASLASLRSVI TETTAHLGVC
 851 DELAAQVSHE DNVLAVVRRE IHGFLSVVSG IHARASKLLS GDQVPGFCFM
 901 GQFLARWRRL SACYQAARAA AGPEPVAEFV QELHDTWKGL QTERAVVVAP
 951 LVSSADQRAA AIREVMAHAP EDAPPQSPAA DRVVLTSRRD LGAWGDYSLG
1001 PLGQTTAVPD SVDLSRQGLA VTLSMDWLLM NELLRVTDGV FRASAFRPLA
1051 GPESPRDLEV RDAGNSLPAP MPMDAQKPEA YGHGPRQADR EGAPHSNTPV
1101 EDDEMIPEDT VAPPTDLPLT SYQ
```

B

(1828)-MC**EAPVVT**REWVL-(1839) **BRCA1**
(9)-**EAPVVT**-(14) **UL37**

Figure R38. The anti-BRCA1 antibody I-20 recognizes UL37 from HSV-1. A) Mass spectrometry analysis identified the reactive band at 120 kDa as the HSV-1 UL37 protein. The sequence coverage observed is 57.8 %, calculated by dividing the number of amino acids in all found peptides (indicated in red) by the total number of amino acids in the entire protein sequence. **B)** The peptide used to generate the anti-BRCA1 antibody I-20 shares six identical amino acids with the HSV-1 UL37 protein at the N-terminal part of the molecule.

Moreover, the mass spectrometry result was complemented by another proteomic approach called Off Gel. This technique does not identify a specific protein but enriches the protein of interest in a sample and identifies the proteins present in the sample. Our result showed that the first HSV-1 protein identified with the highest score was UL37. The best scored protein was UL37, 25 peptides were recognized with a 28.3 %.coverage of UL37.

The mass spectrometry data were confirmed by immunoprecipitation from Vero cells using I-20 followed by western blot with a specific primary antibody targeting UL37 (Klupp, Granzow et al. 2001) (Fig. 39A). The results showed a clear band of the relevant molecular size for its target protein, which had the same molecular mass as the band recognized by I-20. These data suggest that I-20 preferentially binds UL37.

The cross-reactivity of I-20 was further confirmed by infection of VERO cells with an HSV-1 deficient in UL37 (ΔUL37 HSV-1) (Fig. 39B). This experiment showed that I-20 did not detect the 120 KDa-

protein in the lane corresponding to the cells infected with the virus deficient in UL37.

Also, the cross-reactive binding of I-20 to the N-terminus of UL37 was further analysed by transfection of 293T cells with 1) a plasmid containing a GFP-UL37 construct, 2) a plasmid containing only the N-terminus of UL37, which contains the potential cross-reacting motif (the six aminoacid shared with the BRCA1 I-20 antigenic molecule), and 3) a plasmid lacking the N-terminus of UL37 (Fig. 39C). These experiments showed that I-20 recognized the full-length and N-terminal fusion proteins of UL37, but not the fusion protein lacking the N-terminus. Thus, these data confirmed that I-20 binds specifically to the N-terminus region of UL37 as predicted from the epitope mapping.

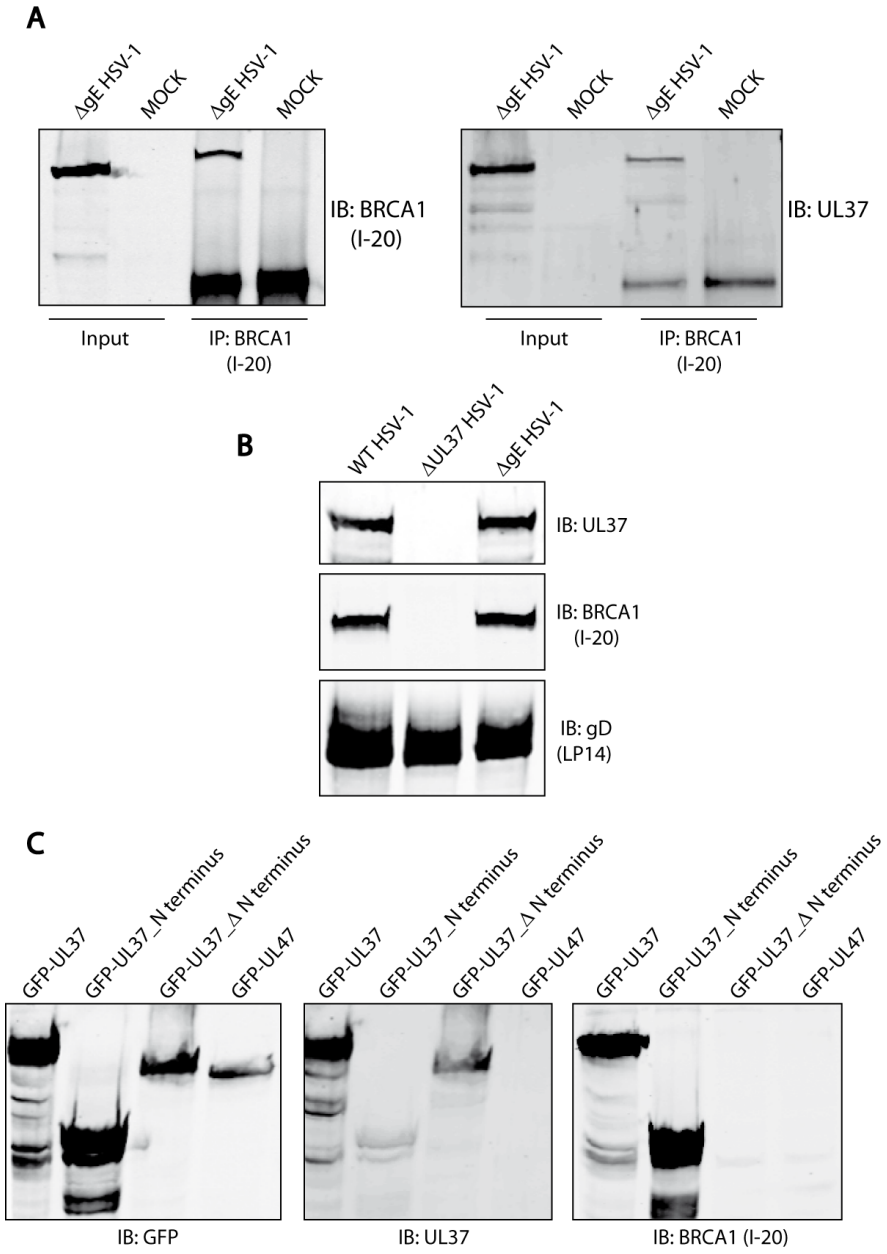


Figure 39. The cross-reactivity of I-20 with HSV-1 UL37 was validated in three ways: (A) by immunoprecipitation of mock infected and ΔgE HSV-1-infected VERO cells using I-20 followed by western blot with specific anti-UL37 or anti-BRCA1 I-20 antibodies. Total lysates (input) were also immunobloted with the same antibodies; **(B)** by infection of VERO cells with an HSV-1 deficient in UL37, a wt HSV-1 or ΔgE HSV-1. Lysates from these cells were immunobloted with specific anti-UL37, anti-BRCA1 I-20 and anti-gD LP14; **(C)** by transfection of 293T cells with a plasmid containing the GFP-UL37 construct, another plasmid containing only the N-terminus of UL37 which contains the potential motif and a plasmid lacking the N-terminus of UL37. Lysates from these cells were immunobloted with anti-GFP, specific anti-UL37 or anti-BRCA1 I-20 antibodies.

Taken together, the above data demonstrated that the **I-20 antibody crossreacts with the tegument protein UL37 from HSV-1**, which is the 120 KDa protein recognized after HSV-1 infection.

3. Study of IFI16 expression in human cancer cells of different p53 genotypes

3.1. Expression of IFI16 in the wild type and p53 deficient HCT116 colorectal carcinoma cell line.

As described in the introduction, in addition to the role in anti-viral defense, IFI16 was first suggested to be linked to tumour suppression. Although it cannot be classified as a conventional tumour suppression protein it satisfies several important criteria. For example, IFI16 has been associated with reduced cell proliferation, cell cycle arrest and cellular senescence. Moreover, it has been observed to interact with several tumour suppressor proteins such as pRb, p53 and BRCA1. Also, IFI16 expression is lost in several human tumours such as breast and prostate cancer cells (Johnstone, Wei et al. 2000; Aglipay, Lee et al. 2003; Xin, Curry et al. 2003; Fujiuchi, Aglipay et al. 2004; Raffaella, Gioia et al. 2004; Xin, Pereira-Smith et al. 2004; Kim, Park et al. 2005).

Regarding the relationship of IFI16 with p53 it is known that IFI16 and p53 associate with each other and that the binding of IFI16 to p53 is important for p53 binding to DNA (Johnstone, Wei et al. 2000). Specifically, the HIN-A domain of IFI16 binds the C-terminus of p53, likely preventing p53 from nonspecific DNA interactions; whereas the HIN-B domain recognizes the core domain of p53 stabilizing the p53-DNA binding (Liao, Lam et al. 2011).
A variety of evidence suggests that the IFI16-p53 interaction enhances p53 transcriptional activity (Johnstone, Wei et al. 2000; Fujiuchi, Aglipay et al. 2004; Xin, Pereira-Smith et al. 2004; Gugliesi, Mondini et al. 2005; Duan, Ponomareva et al. 2011). Moreover, it is also likely that IFI16 is involved in recruitment of additional factors to the p53-binding sites via its PYRIN domain. Indeed, the BRCA1 protein interacts with the PYRIN domain of IFI16 and promotes p53-dependent apoptosis (Aglipay, Lee et al. 2003).

Although a role for IFI16 in modulating p53 activity has been suggested, whether p53 regulates IFI16 expression or activity is still unclear. There is only one report stating that functional activation of p53 can stimulate the transcription of the IFI16 gene in normal human fibroblasts and the human osteosarcoma Saos2 cell line (Song, Alimirah et al. 2008).
To better understand how p53 could regulate IFI16, lysates from HCT116 cells (colorectal carcinoma) expressing wt p53 and HCT116 p53-/- were analysed by western blot using antibodies anti-IFI16, anti-p53 and anti-β-actin as a loading control. Surprisingly a marked decrease in IFI16 protein levels was observed in the p53 deficient cells compared to those in wt HCT116 cells (Fig. R40).

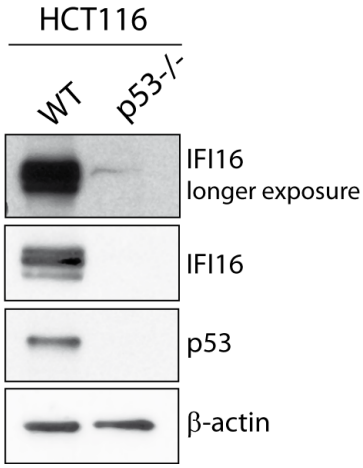


Figure R40. IFI16 is hardly expressed in HCT116 p53$^{-/-}$ cells. 50 µg of total lysates from HCT116 cells, wt and p53$^{-/-}$, were analysed by western blot with antibodies to IFI16, p53 and β-actin.

As described in the introduction, IFI16 protein was firstly considered as a nuclear protein, specifically nucleolar, due to the presence of a nuclear localization sequence. However, several studies have reported that IFI16 can be found in the nucleus, cytoplasm or both compartments depending on the cell type and several other factors (reviewed in (Veeranki and Choubey 2011)). Given that IFI16 protein can associate with other proteins including p53, it is likely that these interactions contribute to the subcellular localization of IFI16. Since p53 has been considered mainly a nuclear protein, although it can also localize in the cytoplasm (reviewed in (O'Brate and Giannakakou 2003)), the interaction between p53 and IFI16 could contribute to the main nuclear localization of IFI16 in some cell types. However, in the absence of p53, IFI16 could be mislocalized to the cytoplasm leading to a reduction in IFI16 stability. To test this hypothesis HCT116 wt and p53-/- cells were subjected to subcellular fractionation and immunoblotting analysis for IFI16 (Fig. R41). The validity of the cell fractionation procedure was confirmed by assay for lamin A/C (a nuclear protein) and MEK1/2 (cytosolic protein). IFI16 was mainly concentrated in the nuclear fraction with relatively lower levels in the cytoplasmic fraction in wt HCT116 cells. As previously observed in p53-null HCT116 cells IFI16 was barely expressed, however, IFI16 was slightly more abundant in the cytoplasmic fraction. This result was consistent with the hypothesis that the association of p53 might affect IFI16 stability.

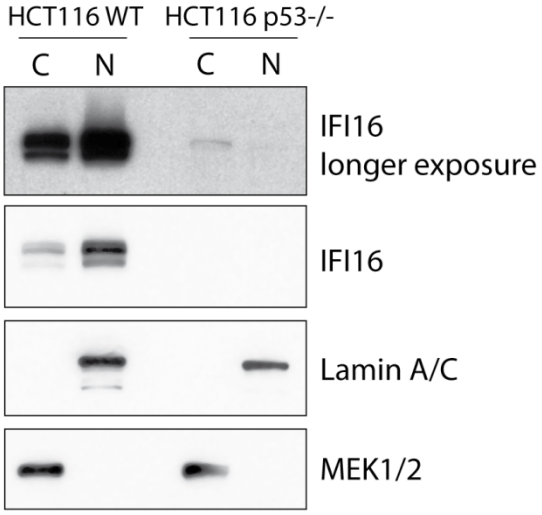


Figure R41. IFI16 is mainly nuclear in wt HCT116 but it is barely expressed in HCT116 p53$^{-/-}$ cells. HCT116 wt and p53$^{-/-}$ cells were subjected to subcellular fractionation. Equal amounts (15µg) of total protein were loaded in each lane and were analysed by western blot with an antibody to IFI16. Lamin A/C was used as a nuclear (N) marker, and MEK 1/2 was used as a cytoplasmic (C) marker.

In spite of the fact that HCT116 cells are tumour-derived cells, they express wt p53. Therefore, the reduced expression of IFI16 in HCT116 p53-/- cells was consistent with the possibility that wt p53 was involved in regulating the stability of IFI16 protein or that wt p53 could regulate IFI16 expression at either a transcriptional or translational level.

If the absence of p53 protein was associated with the marked decrease of IFI16 protein expression due to a role of p53 on IFI16 expression or stability then, the reconstitution of p53 expression could restore IFI16 expression. To test this hypothesis, HCT116 p53-/- cells were transfected with a plasmid containing p53 wt or a plasmid containing a p53 mutant sequence (R175H, an arginine to histidine mutation at residue 175). The R175H mutation is a hotspot mutation in the DNA-binding core domain of p53, classified as a p53 structural mutation, i.e it greatly perturbs the conformation of its DNA-binding region (reviewed in (Sigal and Rotter 2000; Joerger and Fersht 2007)). As with all hotspot mutations, it abolishes the wild type tumor suppression function of p53, but this mutant has also acquired new oncogenic functions in promoting tumorigenesis (Liu, Song et al. 2010). Lysates from transfected HCT116 p53-/- cells were analysed by western blot with antibodies to IFI16, p53 and β-actin (Fig. R42). As shown in Figure R42 no re-expression of IFI16 protein was observed after p53 reconstitution with wt or mutant p53.

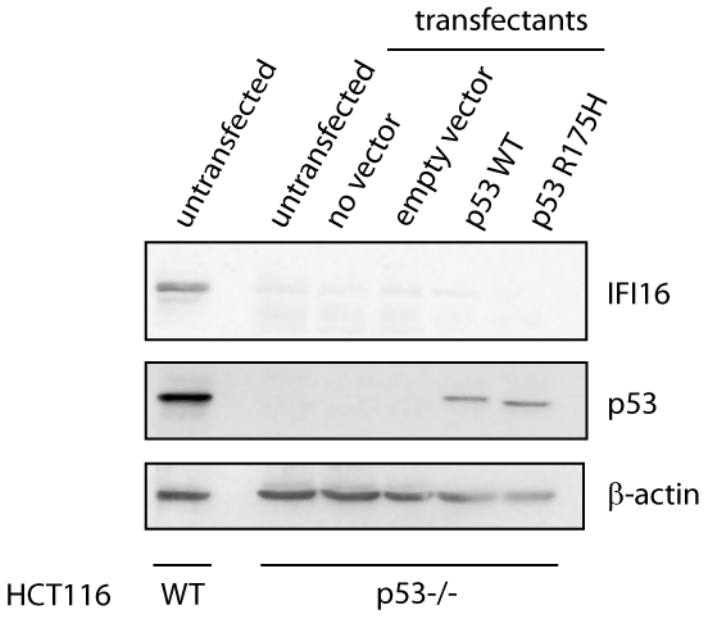


Figure R42. IFI16 expression is not reconstituted in HCT116 p53-/- cells after p53 restoration. HCT116 p53-/- were plated into a 6 well-plate and transfected with 3 μg of empty vector or expression vector for p53 wt or the mutant p53-R175H using jetPEI. HCT116 p53-/- cells were also exposed to jetPEI reagent (no vector) as another negative control. Total cell lysates were prepared at 44 hours after transfection and 25 μg of protein lysates from these cells and from HCT116 wt, as a positive control of IFI16 and p53 expression, were analysed by western blot using antibodies to IFI16, p53 and β-actin.

This result was consistent with a previously published report where the expression of tranfected wt p53 did not affect the expression levels of transfected IFI16 (Johnstone, Wei et al. 2000).

It is well known that p53 regulates the expression of different genes, mainly involved in cell cycle arrest and apoptosis, in response to various forms of cellular stress (reviewed in (Laptenko and Prives 2006; Kruse and Gu 2009)). Moreover, the single report about IFI16 up-regulation by p53 showed that activation of p53, for example in response to certain DNA-damaging agents, stimulated the transcription of the *IFI16* gene (Song, Alimirah et al. 2008).

Therefore to ascertain if activation of p53 was required for regulating IFI16 expression the previous transfections were repeated and 24 hours after transfection cells were subjected to different DNA-damaging agents: short wave ultraviolet radiation (254nm) during 15 minutes as a non-ionizing radiation (Fig. R43) or gamma radiation (Cesium 137,10Gy) as an ionizing radiation (Fig. R44). Total cell lysates were prepared at 4 hours post-irradiation and were analysed with antibodies to IFI16, p53 and β-actin. The cells were collected at this time point because according to Fujiuchi *et al.* in cells in which IFI16 expression is reduced the maximal activation and, in consequence, stabilization of p53 occurred at 2-4 hours post-irradiation (10Gy) (Fujiuchi, Aglipay et al. 2004). Therefore, if IFI16 expression levels were regulated by p53 activation, an IFI16 upregulation might be expected when p53 was maximally activated.

Moreover to assess whether p53 activation could also affect the expression levels of a transfected IFI16, IFI16 isoform B was cloned and transfected together with wt or mutant p53.

As shown in Figure R43, after exposure to gamma radiation IFI16 levels were similar to those of non-irradiated cells in wt HCT116, non-transfected HCT116 p53-/- and p53-transfected cells. Similarly, no differences were found in IFI16 expression levels between non-transfected and p53-transfected HCT116 p53-/-cells after exposure to gamma radiation. Moreover, when IFI16 and p53 were co-expressed in these experiments, the presence of p53 does not seem to increase the levels of transfected IFI16 since they were equivalent between wt and mutant p53-transfected cells. However, in the experiments shown in Figure R43 a control for IFI16 transfection alone would be required to be able to compare the IFI16 expression levels properly. This control was included when UV radiation was used as the DNA-damaging agent (Fig. R44) and it was observed that cotransfection of p53 with IFI16 did not increase the levels of the transfected IFI16 compared to those seen in cells transfected with IFI16 alone. Moreover, after exposure to UV IFI16 levels of cells transfected with p53 were similar to those from non-transfected and non-irradiated p53 null cells. Although in this experiment a control of non-transfected but UV-exposed p53-null cells, as included in Figure R43, would have been required to allow more confident interpretation of the data.

These results indicate that p53 seems not to induce the up-regulation of IFI16 expression after exposure to ionizing and non-ionizing radiation.

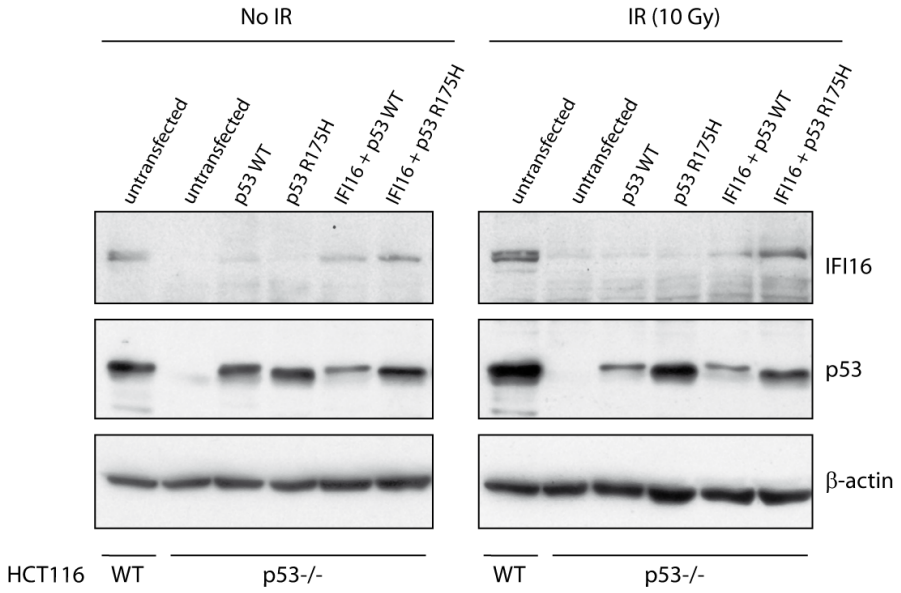


Figure R43. IFI16 expression is not reconstituted in HCT116 p53$^{-/-}$ when p53 is restored and activated after gamma irradiation. HCT116 p53$^{-/-}$ were plated in duplicates into 6 well-plates and transfected with 3 μg of expression vector for p53 wt or the mutant p53-R175H or 1.5 μg of each plasmid in the double transfections (IFI16 full-length plus p53 wt or the mutant p53-R175H) using jetPEI. 24 hours after transfection plates were exposed or not to ionizing radiation (gamma irradiation; source Cesium 137; 10 Gray). 4 hours after irradiation total cell lysates were prepared and 25 μg of protein lysates were analysed by western blot using antibodies to IFI16, p53 and β-actin.

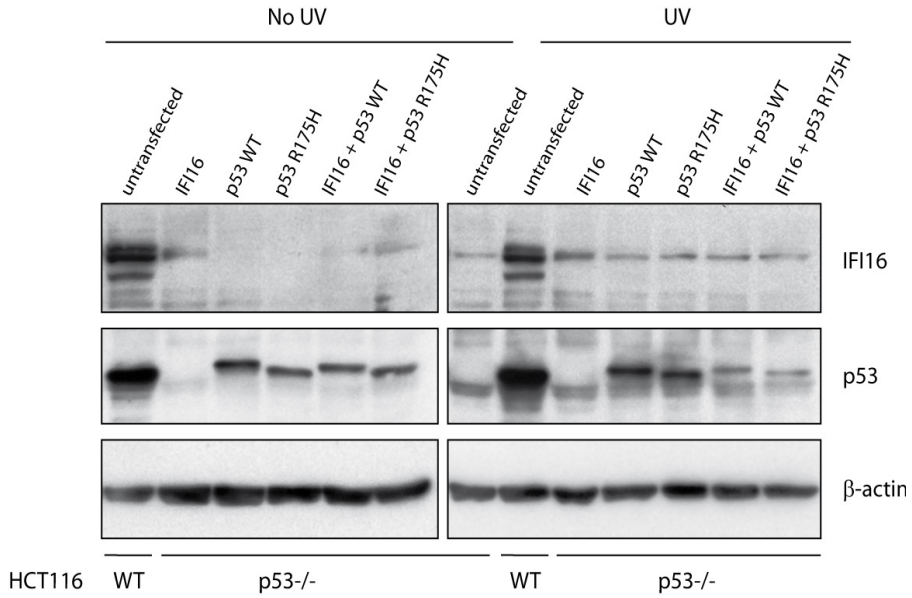


Figure R44. IFI16 expression is not reconstituted in HCT116 p53-/- when p53 is restored and activated after UV radiation. HCT116 p53-/- were plated in duplicate into 6 well-plates and transfected with 3 μg of expression vector for IFI16 full-length, p53 wt or the mutant p53-R175H or 1.5 μg of each plasmid in the double transfections (IFI16 plus p53 wt or the mutant p53-R175H) using jetPEI. 24 hours after transfection plates were exposed or not to UV radiation (254 nm) during 15 minutes. 4 hours after irradiation total cell lysates were prepared and 30 μg of protein lysates from these cells and from HCT116 wt, as a positive control of IFI16 and p53 expression, were analysed by western blot using antibodies to IFI16, p53 and β-actin.

In conclusion, these results suggested that on activation in response to DNA-damaging agents, p53 was not able to induce the expression of IFI16. However, inclusion of a control for p53 activation by both types of radiation, for example p53-phosphorylated on Serine15, would provide more confidence in this conclusion. Even though gamma (ionizing radiation) and UV (non-ionizing radiation) exposure induce different types of DNA damage (ionizing radiation induces DNA strand breaks whereas UV light provokes pyrimidine dimers), and distinct signalling pathways are triggered following exposure to these different types of radiation (reviewed in (Ljungman 2000; Ljungman and Lane 2004)), phosphorylation of Serine15 is a major focal point in the activation of p53 during both DNA damage responses (Loughery, Cox et al. 2014). Specifically, this residue is phosphorylated in an ATM/ATR-dependent manner in response to IR, and in an ATR-dependent manner in response to UV (reviewed in (Lakin and Jackson 1999)).

Thus, although these preliminary data do not allow rejection of the hypothesis that p53 could have a role in IFI16 expression in HCT116 cells, it seems that p53 activation is not sufficient to activate IFI16 expression.

We had also planned to measure the IFI16 mRNA levels of wt and p53-null HCT116 cells by quantitative real-time PCR (qRT-PCR) to assess if the regulation of IFI16 by p53 could be at mRNA or protein level. For example, if the expression of the corresponding IFI16 mRNA was not affected in the p53 knockout cells compared to that from wt cells, any IFI16 protein up-regulation observed in wt cells might be a result of protein accumulation through IFI16 stabilization by p53 or p53 could be involved in IFI16 translation. On the other hand, if the levels of IFI16 mRNA were down-regulated in the p53-null cells, this could indicate that p53 could stimulate the IFI16 transcription. Nevertheless, it would be essential to verify the integrity of *IFI16* gene in HCT116 p53-null cells.
However, it proved impossible to continue doing more experiments with the HCT116 cells because both wt and p53 knockout cells, started to grow as spheroids instead of as a monolayer. For as yet unknown reasons, when the wt cells formed these spheroids they did not express IFI16 anymore. Intriguingly, no other batches of HCT116 cells supplied by other groups expressed IFI16 protein.

3.2. Expression of IFI16 protein in human mammary cell lines

We next wanted to extend the analysis of IFI16 expression in other cell lines of known p53 genotype. As mentioned before it is known that levels of IFI16 mRNA and/or protein are frequently decreased in many breast cancer cell lines and tissues from breast cancer patients (Fujiuchi, Aglipay et al. 2004), suggesting a potential link between loss of IFI16 and breast cancer development. Although other factors apart from p53 could affect IFI16 expression in these cells, we wanted to take advantage of the extensive knowledge of p53 genotype in breast cancer cells to study its possible relation with

IFI16 expression.

Therefore to relate the genotype of p53 to the IFI16 expression levels, lysates from different human mammary cell lines (normal and tumoral) (Table R3) were analyzed by western blot with antibodies to IFI16 and p53 (Fig. R45).

In order to establish the p53 mutations in the breast cell lines used in our study, we compared the information among different databases which describe the p53 status in cell lines: the p53 database that was originally developed by T. Soussi at the Institut Curie in Paris, France (http://p53.free.fr/) and the International Agency for Research on Cancer *TP53* database (http://p53.iarc.fr/) maintained and curated in Lyon, France. In addition, we contrasted the previous information with the Catalogue of Somatic Mutations in Cancer (COSMIC) database of the Sanger Institute, England (http://cancer.sanger.ac.uk/cosmic). This database is not specific to p53 mutations but it does tabulate multiple somatic mutations and related published information relating to human cancers and cancer cell lines.

During the last years there has been an extraordinary increase in the information about DNA sequence mutations and in particular there is a vast amount of information about p53 mutations, which are the genetic defects most frequently reported in human cancers (reviewed in (Levine and Oren 2009; Olivier, Hollstein et al. 2010)). For this reason different databases registering all published p53 mutations have been established. Although the different databases show slight differences in some cell lines due to a lack of homogeneous data in the literature, to our knowledge the IARC online resource claims to track reported p53 mutations and performs updated data retrieval as well as reviewing and editing the database contents. Moreover this database contains the full reference of all publications describing the p53 mutations in each cell line. In addition, the p53 mutations registered in the IARC database are in concordance with our results (Fig. R45). Therefore, we used mainly the data of p53 mutations from the IARC database (Table R4) in order to relate IFI16 expression to the p53 genotype.

Wild type p53	Mutant p53
MCF10A (Normal)	MDA-MB-468 (Tumoral)
HB4a (Normal) (n/a)	BT-474 (Tumoral)
MCF7 (Tumoral)	SK-BR-3 (Tumoral)
	MDA-MB-361 (Tumoral)
	MDA-MB-453 (Tumoral)
	T-47D (Tumoral)
	Hs578T (Tumoral)
	BT-549 (Tumoral)
	MDA-MB-157 (Tumoral)*
	MDA-MB-231 (Tumoral)*

Table R3. p53 status of the breast cell lines used in this study. Although there is no information available about the status of p53 genotype in HB4a, it was classified in the wt p53 group because it is a nontumorigenic and nontransformed breast cell line. n/a: not available. (*) MDA-MB-157 and MDA-MB-231 cell lines were later shown to be actually the colon cell line SW480.

Note that although the MDA-MB-231 and MDA-MB-157 cell lines appear in Table R3 and Figure R45-C, the provenance of these cancer cell lines was not clear and they actually turned out to be the colon cell line SW480 when cell genotyping was performed. For this reason the p53 mutations of these cells do not appear in Table R4.

Cell line	Nucleotide change	Aminoacid change
MDA-MB-468	c.818G>A	p.R273H
BT-474	c.853G>A	p.E285K
SK-BR-3	c.524G>A	p.R175H
MDA-MB-361	c.166G>T	p.E56*
MDA-MB-453	c.1101_1130del30 / c.991-1182del192	p.?
T47D	c.580C>T	p.L194F
Hs-578T	c.469G>T	p.V157F
BT-549	c.747G>C / c.108G>A	p.R249S / p.P36P

Table R4. p53 mutations of the breast cancer cell lines used in this study according to the International Agency for Research on Cancer (IARC) TP53 Database. (*) means STOP codon.

It is important to note that the breast cancer cell lines analysed which possess a missense mutation (MDA-MB-468, BT-474, SK-BR-3, T-47D, Hs578T and BT-549), in contrast to cell lines with deletion (MDA-MB-453) and nonsense (MDA-MB-361) mutations, displayed elevated levels of p53 protein as compared to the wt protein (Figure R45). As mentioned in the first section of Results, in normal cells p53 has a very short half-life because wt p53 is degraded by MDM2-mediated proteolysis. However, mutated p53 often has an extended half-life due to its defect in MDM2 transactivation and binding. Missense mutations are the most frequent genetic alterations in *p53* and most of them expand p53 half-life producing an accumulation of the full-length protein (reviewed in (Brosh and Rotter 2009)). For this reason accumulation of mutant p53 is a common characteristic of human cancer, including breast tumours (Bartek, Bartkova et al. 1991). Therefore our result agreed with previous reports showing that many human breast tumours accumulated high levels of a missense mutant p53 (Bartek, Bartkova et al. 1990; Bartek, Iggo et al. 1990; Davidoff, Humphrey et al. 1991).

Unlike wt HCT116, not all the breast cell lines that have wt p53 (MCF10A, HB4a and MCF7) express IFI16; only the normal cell lines MCF10A and HB4a expressed IFI16 (although note that HB4a only expressed one of the three IFI16 isoforms), whereas the tumoral MCF7 did not express IFI16. However, the p53 molecule expressed in MCF7 ran slightly slower than the p53 protein from MCF10A (Fig. R45 A-B).

On the other hand, most of the breast cancer cell lines that have a mutant p53 showed no IFI16 expression, except Hs578T, which expressed IFI16 levels comparable to MCF10A, and BT-549, which expressed slight levels of IFI16 (Fig. R45 A,B and C).

These results were consistent with the previous report indicating a frequent loss of IFI16 in many human breast cancer cells (Fujiuchi, Aglipay et al. 2004), thus reinforcing the idea of an involvement of IFI16 in tumour suppressor functions in breast cancer.

Although these results were consistent with the previously published data, no direct correlation between the presence of functional p53 and the expression of IFI16 in breast cell lines as shown in HCT116 was observed. It seems that the expression of IFI16 may be more dependent on the cellular phenotype (normal or tumoral) in breast cells.

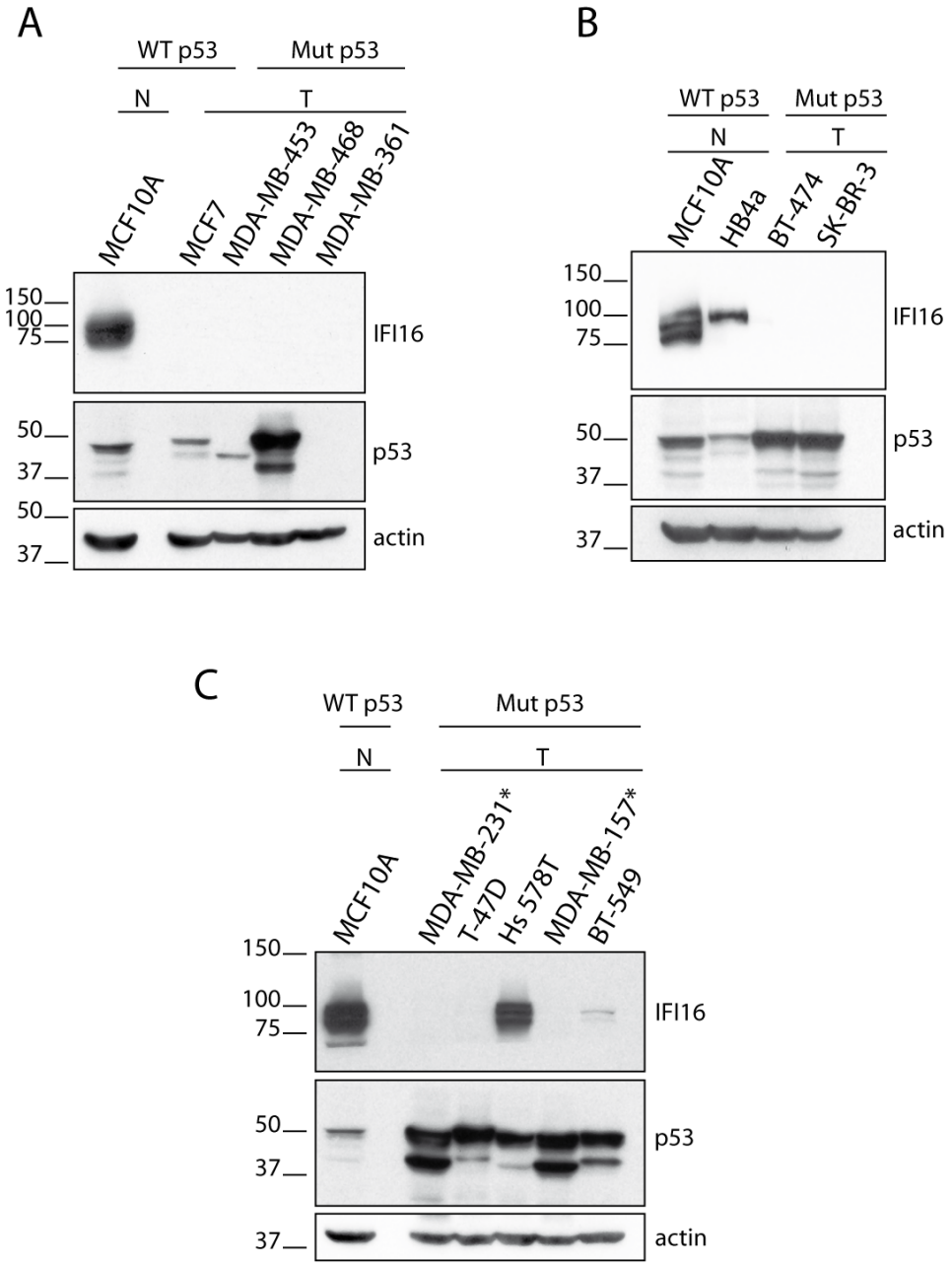


Figure R45. Analysis of IFI16 expression based on p53 status in human breast cells. (N) Normal breast cell lines and (T) tumor breast cell lines. 50 µg of total lysates from different breast cells lines were analysed by western blot with antibodies to IFI16, p53 and β-actin. (*) MDA-MB-157 and MDA-MB-231 cell lines were later shown to be actually the colon cell line SW480.

Taken together, these results suggested that IFI16 function could be lost in breast cancer development.

3.3. Expression of IFI16 expression in human bladder cell lines

As mentioned previously, several studies and our own work have demonstrated that loss or reduced expression of IFI16 is often associated with breast cancer (Fujiuchi, Aglipay et al. 2004), and other human cancers such as prostate cancer (Xin, Curry et al. 2003).

To study if the reduction of IFI16 expression was consistent in other epithelial cancer cell lines not yet studied for IFI16 expression, lysates from a panel of different bladder tumour cell lines (Table R5) that have different mutational status of p53 (Table R6) were analysed by western blot using antibodies to IFI16 and p53 (Fig. R46).

Wild type p53	Mutant p53
RT4	RT-112
	J82
	UM-UC-3
	T-24

Table R5. p53 status of the bladder tumor cell lines used in this study.

Cell line	Nucleotide change	Aminoacid change
RT-112	c.548C>G / c.743G>A	p.S183* / p.R248Q
J82	c.811G>A	p.E271K
UM-UC-3	c.338T>G	p.F113C
T24-A	c.378C>G	p.Y126*

Table R6. p53 mutations of the bladder cancer cell lines used in this study according to the International Agency for Research on Cancer (IARC) TP53 Database and/or Lopez-Knowles et al. (*) means STOP codon.

It is important to note that according to the three databases that we checked for p53 mutational status, p53 could contain different mutations in the J82 cell line due to a controversy between publications. Moreover, there are two sublines of the T24 cell line which seem to contain a different p53 mutation. In these experiments we have assumed that the p53 mutations in both cell lines (shown in Table R6) were the ones defined in a mutational analysis carried out by the group that provided us with these cells (Lopez-Knowles, Hernandez et al. 2006).

As shown in Fig. R46 IFI16 expression was detected in all the bladder tumour cell lines independently of the p53 status. Indeed, the RT4 cell line that contains wt p53 expressed lower levels of IFI16 than the other cell lines. Unlike the majority of the breast cancer cell lines analysed, a direct correlation between the absence or reduction of IFI16 expression and tumoral phenotype was not observed in the bladder cancer cell lines studied. Moreover, a relation between the presence of functional p53 and the expression of IFI16, as we observed in HCT116 cells, was not found in these bladder tumour cells.

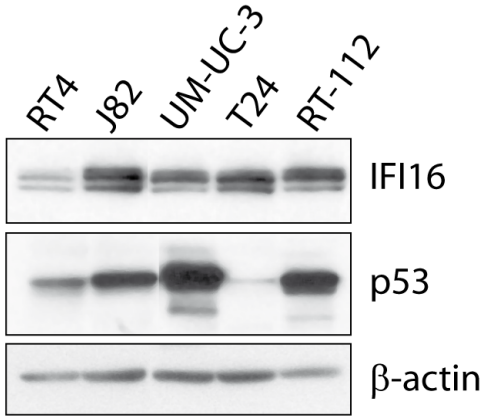


Figure R46. Analysis of IFI16 expression based on p53 status in bladder tumour cell lines. 40 μg of total lysates from different bladder tumour cell lines were analysed by western blot with antibodies to IFI16, p53 and β-actin.

Altogether, the results shown here demonstrated that IFI16 is never expressed in the absence of p53 in HCT116 cells. This is consistent with the hypothesis that p53 expression is required but not sufficient for the expression of IFI16 in HCT116 cells, because the transfection with p53 was not sufficient to activate IFI16 expression. The nature of the other factors required for IFI16 expression remains unclear. When the expression of IFI16 protein in eleven breast cell lines was analyzed, it was apparent that the majority of the cells that have a mutant p53 (all except Hs578T) did not express or slightly expressed IFI16, however this correlation was not observed in the bladder cancer cell lines studied.

All in all, these results suggest that IFI16 expression regulation by p53 could be tissue-dependent.

Discussion

The importance of innate immune system in response to viral infections is reflected by the complexity of the interactions between the virus and host. Infection with viruses leads to the release of viral DNA into the cellular cytoplasm or nucleus. The cascade of signals activated by microbial DNA sensors not only stimulates the recruitment of immune cells to the site of inflammation, but also could induce changes in the infected cell to facilitate direct immune recognition of the infected cell (Hamerman, Ogasawara et al. 2004; Kloss, Decker et al. 2008). Therefore it is not surprising that viruses have developed strategies to evade the recognition and signaling by DNA sensors. Several DNA sensors have been identified, among them IFI16, which on recognition of viral DNA induces the production of type I IFN and the assembly of the inflammasome complex leading to maturation of inflammatory cytokines (Unterholzner, Keating et al. 2010; Kerur, Veettil et al. 2011). Recently, IFI16 has been shown to play an additional role in antiviral defense by silencing herpesvirus genomes, specifically HSV-1 and HCMV genomes (Orzalli, Conwell et al. 2013) (Gariano, Dell'Oste et al. 2012; Johnson, Bottero et al. 2014).

During experiments involving virus infection it was observed that HSV-1 infection led to a loss of IFI16 expression. An initial hypothesis was that the reduced expression of these molecules reflected a general inhibition of synthesis of cellular proteins associated with virus infection, however other observations suggesting that IFI16 has a long half-life in the cell (data not shown) indicated that this hypothesis was not correct and suggested the existence of viral strategies to evade the immune response promoted by this molecule via specific inhibition of its expression. Indeed, Orzalli *et al*. concluded that ICP0 promoted the IFI16 degradation in a proteasome- and ICP0 RING finger domain-dependent mechanism. However, conflicting data have been published on the role of ICP0 in mediating destabilization of the DNA sensor protein IFI16 after HSV-1 infection. Thus, the impact of ICP0 on IFI16 remains to be fully understood. In this thesis this phenomenon has been re-examined and these data confirm that infection with HSV-1 leads to a marked reduction in IFI16 expression levels, although the extent of the loss of IFI16 varies somewhat between different cell lines. These data also show that IFI16 levels are slightly reduced after infection with ICP0 mutant virus, but that the reduction in IFI16 expression is markedly enhanced if the HSV-1 expresses ICP0 and that this loss of IFI16 can occur even in the absence of efficient viral replication.

The existence of an ICP0-dependent regulation of IFI16 stability after HSV-1 infection was first suggested by the observation that the decrease in IFI16 seen after infection with wt HSV-1 (strain KOS) or a virus which expresses ICP0 but not additional IE gene products (*d*106) is markedly greater than that observed after infection with a virus which expresses no IE proteins (*d*109) (Orzalli, DeLuca et al. 2012; Johnson, Chikoti et al. 2013). However, a third paper, from a group with considerable expertise in the biology of ICP0, concluded that ICP0 was neither necessary nor sufficient to trigger a loss of IFI16 (Cuchet-Lourenco, Anderson et al. 2013). They argued that ICP0 controls the likelihood of progression of viral infection (Everett, Boutell et al. 2004) and that the efficient entry into lytic replication, rather than ICP0 expression *per se*, regulates IFI16 stability (Cuchet-Lourenco, Anderson

et al. 2013). This argument was supported by experiments showing that IFI16 can be degraded normally in the absence of ICP0 as long as the lytic cycle is progressing efficiently (Cuchet-Lourenco, Anderson et al. 2013). We could not achieve the same infection progression in both ICP0-null and control virus infections, as did Cuchet-Lourenço and colleagues, to compare properly the levels of IFI16 between the two infections, and this could be explained due to the differences in the moi used, since variations in the multiplicity could provoke modifications in the results. However, the key observation of this thesis is that infections with a set of mutant viruses deficient for expression of one or more IE genes including ICP4 but excluding ICP0, and thus unable to complete a normal cycle of viral replication(DeLuca, McCarthy et al. 1985), as judged by UL42 expression, are sufficient to produce a marked decrease in IFI16 (Fig. R4). These viruses will express only very low levels, if any, of HSV-1 viral gene products other than ICP0 and so these data clearly show that ICP0 expression, even in the context of a non-productive viral infection, can be sufficient to trigger a marked reduction of IFI16 expression levels. It is not clear why expression of ICP0 during a viral infection, but not by transfection, should be sufficient to trigger IFI16 loss but this might be related to the levels of ICP0 expression. ICP0 has been reported to be expressed at higher than normal levels in cells infected with ICP4 mutant viruses (DeLuca, McCarthy et al. 1985), while the levels of ICP0 achieved in the induced cells used by Cuchet-Lourenço *et al*, although sufficient to trigger PML degradation, were lower than those obtained after infection with wt HSV-1 strain 17 (Cuchet-Lourenco, Anderson et al. 2013). Specifically, the levels of ICP0 induced in the cell system employed by these authors were equivalent to those at early stages of infection. These data suggested that the amount of ICP0 needed to degrade IFI16 was higher than that for PML degradation. Therefore, further quantitative studies comparing the expression levels of ICP0 between cell lines and their impact on IFI16 levels would be an important area for further investigation. In this context, a control for ICP0 expression in all of our western blot analyses would have been convenient. The differences between PML and IFI16 degradations might also reflect the use, by ICP0, of distinct mechanisms to target both proteins. Since ICP0 uses a variety of mechanisms to target different substrates for degradation, elucidation of the specific mechanism employed by ICP0 to target IFI16 would be an interesting area of study.

Viral proteins that enter the cells as part of the virion might interact with ICP0 to produce the reduction in IFI16, but this explanation seems unlikely since cells exposed to the *d109* virus that contains mutations in the five IE genes ICP0, ICP4, ICP22, ICP27, and ICP47, effectively blocking all viral gene expression during infection (Samaniego, Neiderhiser et al. 1998) does not trigger a loss of IFI16 expression, although this virus lacks ICP0 (Orzalli, DeLuca et al. 2012).

The simplest model that can be advanced to reconcile these observations is that the turnover of IFI16 protein depends on cellular factors, but that this process is enhanced in the presence of ICP0. In this model HSV-1 infection, for example by shutting-off host-protein synthesis, will lead to a reduction in IFI16 expression at a rate that depends on the stability of IFI16 in that particular cell. However expression of ICP0 acts to “catalyze” this loss of IFI16.

The idea that cellular factors influence the loss of IFI16 after HSV-1 infection is mainly based

on the observation that the degree to which IFI16 expression is reduced after infection varies between cell lines. For example, in both our experiments and those of Cuchet-Lourenço et al (Cuchet-Lourenco, Anderson et al. 2013) infection of the U2OS osteosarcoma cell line was associated with lower levels of IFI16 loss than seen after HSV-1 infection of HFFs. Although in our experiments a more pronounced loss of IFI16 was observed in U2OS cells infected with the control virus than that described by Cuchet-Lourenço and colleagues. Some of this variation may reflect differences in experimental conditions; in our experiments IFI16 expression was analysed 24 hpi whereas they analysed IFI16 12 hpi (Cuchet-Lourenco, Anderson et al. 2013). Although cell death may contribute to the decreased IFI16 levels at 24 hpi, analysis of the expression of other cellular proteins would have confirmed that IFI16 is specifically targeted for degradation during HSV-1 infection. However, other published studies have determined the kinetics of IFI16 degradation showing a progressive loss of IFI16 that directly depends on the time of infection (Orzalli, DeLuca et al. 2012; Cuchet-Lourenco, Anderson et al. 2013; Johnson, Chikoti et al. 2013), with little or no IFI16 staining by 24 hpi (Johnson, Chikoti et al. 2013).

An interesting observation is that IFI16 degradation during infection of HFF, U2OS and Vero cells with the replication-defective mutant viruses deleted for the regulatory IE genes except ICP0 correlates with the number of IFI16 isoforms observed in each cell line (Fig. R3). One hypothesis to explain this observation could be that expression of the cellular factors that regulate the expression of the different IFI16 isoforms or their turnover might vary among different cell lines, thereby affecting the efficiency of IFI16 degradation mediated by ICP0.

Indeed, inspection of data from a number of other authors confirms that there is considerable variation in the effect of HSV-1 infection on IFI16 between different cell lines. HSV-1 infection of HEp-2 cells (derived from an epithelial carcinoma) produced a loss of IFI16 expression that was moderately more marked than that observed after HSV-1 infection of HEL cells (human embryonic lung cells) (Kalamvoki and Roizman 2014). However, when STING, an interaction partner of IFI16, was deleted, the pattern of IFI16 expression was completely different in both cell lines. Infection of the human mammary epithelial cell line 184B5 leads to an almost complete loss of IFI16 expression after only 4 hpi, whereas infection of another breast cancer cell line, HCC1937, actually leads to accumulation of IFI16 (Dutta, Dutta et al. 2015). The simplest interpretation of all these data is that the stability of IFI16 is regulated by cellular factors differentially expressed between these different cell lines. The identity of these factors is as yet unknown, but IFI16 protein is known to interact with multiple cellular proteins including BRCA1, p53 and cGAS (Johnstone, Wei et al. 2000; Aglipay, Lee et al. 2003; Song, Alimirah et al. 2008; Liao, Lam et al. 2011; Orzalli, Broekema et al. 2015; Diner, Li et al. 2015a). Interestingly, the breast cancer cell line HCC1937 expresses a truncated BRCA1 protein (Chen, Silver et al. 1998; Tomlinson, Chen et al. 1998). Given that BRCA1 has E3 ubiquitin ligase activity, the absence of the wt BRCA1 protein could prevent the ubiquitination of IFI16 leading to its accumulation during HSV-1 infection. Moreover, since the HCC1937 cells are also deficient in functional p53 (Tomlinson, Chen et al. 1998), speculatively, the lack of IFI16 degradation during HSV-1 infection could also be due to the absence of wt p53. It is known that the absence of cGAS is associated with a decreased stability

of IFI16 (Orzalli, Broekema et al. 2015). As Sun and colleagues suggested (Sun, Wu et al. 2013) it is possible that not all cell types express the same repertoire of DNA sensors. Thus cell types with lower expression of cGAS could show a decreased stability of IFI16. Although it has been demonstrated that cGAS promotes the stability of IFI16 in HFF cells, the mechanism by which cGAS enhances IFI16 stability has not been described. As Orzalli *et al* proposed the interaction of cGAS with IFI16 could affect the conformation of IFI16 preventing the binding of an E3 ubiquitin ligase or facilitating the binding of a deubiquitinase (Orzalli, Broekema et al. 2015). IFI16 is also known to undergo multiple post-translational modifications, including acetylation and phosphorylation (Li, Diner et al. 2012) that regulate the intracellular localisation of this protein, but the effects of these modifications on IFI16 stability, nor how they vary between cell lines, are not yet clear. Thus, in addition to the well known cell-type dependent restriction of replication of ICP0 deficient viruses the nature of the cell line used can profoundly affect the experiment, presumably via variation in the expression of other factors that modify or interact with ICP0. Indeed during the writing of this thesis a recent study has also proposed that ICP0 is necessary but not sufficient for IFI16 degradation, suggesting that in addition to the ICP0 ubiquitin ligase activity other viral or cellular mechanisms contribute to IFI16 stability (Diner, Lum et al. 2015b). Finally, in this context, it is interesting to note that STING, which interacts with IFI16, is stable in cancer-derived HEp-2 and HeLa cells infected with wt HSV-1, but degraded when cells are infected with ICP0-null mutants (Kalamvoki and Roizman 2014). Curiously, however, in normal tissue-derived HEL or HEK293T cells, STING is stable throughout infection with either wt or ICP0 mutant viruses. The degradation of STING does not appear to be related to the effects of viral infection on IFI16 degradation in neither case (Kalamvoki and Roizman 2014). Kalamvoki *et al*. concluded that HSV-1 has not evolved the capacity to make STING dysfunctional or to degrade it because STING is not only detrimental for the infection but also necessary for optimal viral replication. Another example of this double functionality related to the IFN pathway would be NF-κB, which could be both deleterious and essential for HSV-1 replication (Taddeo, Luo et al. 2003; Taddeo, Zhang et al. 2004). ICP0 in particular can inhibit NF-κB activation by promoting the degradation of the p50 component of NF-κB (Zhang, Wang et al. 2013), but on the other hand, ICP0 also ubiquitinates IκBα, which is complexed with NF-κB, leading to the activation of NF-κB, thereby stimulating the transcription of NF-κB target genes (Diao, Zhang et al. 2005). Curiously, NF-κB has been shown to be recruited to ICP0 promoter, enhancing the transcription of ICP0 (Amici, Rossi et al. 2006). However, it seems that this is not case for IFI16, which is degraded in almost all the cell lines analyzed, although the degree of loss of IFI16 varies considerably between different cell lines.

In view of these findings, it is tempting to conclude that **ICP0 is necessary for efficient IFI16 degradation**, and depending on the cell type it could also be sufficient to induce IFI16 loss. However, further experiments would be necessary in order to clarify this issue. Although it has been established numerous associations of IFI16 with viral and cellular proteins during HSV-1 infection (Diner, Lum et al. 2015b), further studies will be required to determine the important factors regulating IFI16 stability.

The fact that IFI16 can recognize the presence of viral DNA, specifically during KSHV (Kerur, Veettil et al. 2011; Singh, Kerur et al. 2013), EBV (Ansari, Singh et al. 2013), HCMV (Cristea, Moorman et al. 2010; Li, Chen et al. 2013) and HSV-1 (Unterholzner, Keating et al. 2010; Orzalli, DeLuca et al. 2012; Li, Diner et al. 2012a; Johnson, Chikoti et al. 2013) infections, and exerts antiviral functions also in this compartment has challenged the model in which the presence of DNA in the cytosol is the only factor that determines the difference between self and exogenous DNA (reviewed in (Unterholzner and Bowie 2011; Paludan and Bowie 2013) and has raised the question of how IFI16 distinguishes between viral and cellular DNA. Specially, the recognition of herpesviruses, dsDNA viruses that replicate in the nucleus of infected cells, is of particular interest.

It has long been known that IFI16 binds DNA. Specifically, IFI16 can recognize ssDNA, dsDNA and also RNA (Dawson and Trapani 1995b; Johnstone, Kerry et al. 1998a; Yan, Dalal et al. 2008). Additionally, IFI16 was suggested to bind nonspecifically to GC-rich ssDNA (Yan, Dalal et al. 2008) and GC-rich dsDNA (Luu and Flores 1997). It also bound to the G-rich fragment in the p53 and cMYC gene promoters. Moreover these fragments possessed non-B DNA structures (Egistelli, Chichiarelli et al. 2009). In addition to binding GC-rich regions, some reports have demonstrated a strong preference of IFI16 protein binding to cruciform structure and supercoiled (sc) DNA (Brazda, Coufal et al. 2012). And the crystal structures of the HIN domains from IFI16 in complex with the B-form dsDNA, derived from the vaccinia virus genomic repeat sequences, revealed a non-sequence-specific DNA recognition through electrostatic attraction between the positively charged HIN domain residues and the dsDNA sugar-phosphate backbone (Jin, Perry et al. 2012). Given that the DNA-sensing activity of IFI16 seems to be independent of the DNA sequence (Unterholzner, Keating et al. 2010; Jin, Perry et al. 2012), an alternative explanation proposed by Johnson et al. is that during the circularization of the dsDNA genome of HSV-1 in the host nucleus, a DNA structure that resembles cruciform or supercoiled is recognized by IFI16 (Johnson, Chikoti et al. 2013). Since during lytic infection, HSV-1 genome associates with histones but not in regular repeating nucleosomes as cellular DNA (Kent, Zeng et al. 2004), another possibility could be that HSV-1 genome is more accessible for IFI16 binding. If this were true, IFI16 recognition of viral DNA should mainly occur at late times postinfection since it has been reported that early in infection HSV-1 genome associates with histones but the proportion of viral DNA associated with histones decreases at later times postinfection (Cliffe and Knipe 2008; Oh and Fraser 2008). However, IFI16 actually responds very quickly to the invasion of HSV-1 genomes (Horan, Hansen et al. 2013; Johnson, Chikoti et al. 2013; Everett 2015). Alternatively, IFI16 may recognize the viral genome as damaged DNA. As stated in the introduction, prior to its identification as an innate immune sensor of pathogenic DNA, IFI16 had been shown to interact with components of the DNA damage response (DDR) such as BRCA1 and p53 (Johnstone, Wei et al. 2000; Aglipay, Lee et al. 2003; Song, Alimirah et al. 2008; Liao, Lam et al. 2011). Specifically, IFI16 is known to associate with BRCA1 at sites of genomic DNA damage to induce p53-dependent apoptosis (Aglipay, Lee et al. 2003). Many viruses induce the same DDR pathways as those stimulated by abnormalities in genomic DNA. Some of these viruses including HSV-1 have evolved mechanisms to inhibit, circumvent or exploit the DDR for

example by activation of certain repair proteins or by targeting others for degradation or mislocalization (reviewed by (Lilley, Schwartz et al. 2007)). Consistent with the proposed link between the DDR and innate immune responses to viral DNA, UV-mediated DNA damage promoted the nuclear export of IFI16 (Costa, Borgogna et al. 2011), similar to the IFI16 translocation to the cytoplasm that occurs during certain viral infections (Kerur, Veettil et al. 2011; Ansari, Singh et al. 2013; Johnson, Chikoti et al. 2013; Dutta, Dutta et al. 2015). Moreover, some crucial components of the DDR pathways, such as DNA-PK and MRE11, have also been involved in STING-dependent responses to cytoplasmic or viral DNA (Zhang, Brann et al. 2011a; Ferguson, Mansur et al. 2012; Kondo, Kobayashi et al. 2013). It is also known the antiviral role of p53 by enhancing type I IFN responses (reviewed in (Rivas, Aaronson et al. 2010)) and it has also been shown that the DDR can induce the production of IFN (Brzostek-Racine, Gordon et al. 2011).

Since firstly IFI16 and later PML respond very rapidly to the invasion of HSV-1 (Everett 2015), and in turn IFI16 is related to BRCA1 and p53 during the DDR, this could indicate that IFI16 links the responses against HSV-1 infection: the recruitment of ND10 proteins and the recruitment of DDR proteins. And this highlights the connection between the functions of IFI16.
In the light of these various lines of evidences, it was possible that IFI16 might interact with HSV-1 genome through other DDR proteins such as BRCA1 and p53 as proposed by Johnson *et al*. Since IFI16 is degraded by ICP0, the levels of these proteins could also be hypothesized to decrease during HSV-1 infection, if they are interacting directly with IFI16 in the same complex to bind HSV-1 DNA. Therefore, the expression levels of these proteins were analysed after HSV-1 infection.
Our results show that HSV-1 infection does not induce a decrease in p53 levels via ICP0. Previous publications have demonstrated that in spite of p53 ubiquitination mediated by ICP0, p53 levels were stabilized during HSV-1 infection of HFF-2 cells (Boutell and Everett 2003; Boutell and Everett 2004; Boutell, Canning et al. 2005). Consistent with these findings, we observed that in HFF cells the p53 levels were stabilized after HSV-1 infection in a manner that was not exclusively dependent on ICP0, although its expression could have an impact on p53 stabilization (Fig. R7). Probably the requirement for ICP0 is related to its ability to induce viral gene expression, which triggers cellular responses that inhibit ubiquitination and turnover of p53, as proposed by the group of Roger Everett (Boutell and Everett 2004). Therefore, although HSV-1 infection can lead to the phosphorylation and stabilization of p53 in ATM-dependent (Shirata, Kudoh et al. 2005) and ATM-independent pathways (Boutell and Everett 2004), the ubiquitination of p53 mediated by ICP0 could prevent p53 downstream signaling reducing the levels of Mdm2 and p21, thereby blocking the main mechanism for apoptosis and ensuring the progression of infection, as Shirata and colleagues have suggested (Shirata, Kudoh et al. 2005).

The p53 stabilization was cell-dependent since the work in this thesis showed, as has previously been observed (Boutell and Everett 2004), that p53 was not stabilized during HSV-1 infection of U2OS cells; indeed the total levels of p53 were reduced independently of ICP0 (Fig. R8).

Analysis of the effect of ICP0 on BRCA1 levels during HSV-1 infection revealed that infection with both the ICP0-null virus and the control revertant virus induced a marked increase of an approximately 120-KDa protein reactive with an antibody for BRCA1 (I-20, Santa Cruz Biotechnology) (Fig. R9). This result was surprising since full-length BRCA1 protein has an apparent molecular weight on SDS-PAGE of around 220-230 KDa, however, no protein of this molecular weight was observed in our western blot analysis. Therefore, the ability of HSV-1 to regulate BRCA1 expression levels could not be validated.

These experiments were performed to assay the effects of HSV-1 infection on IFI16, p53 and BRCA1 expression, however, it should be noted that this approach does not allow definition of whether IFI16 binds DNA alone or in complex with other proteins, or whether the DNA-binding activity of IFI16 is involved in the recruitment of other proteins that interact with IFI16 to sites associated with HSV-1 genomes. The degradation of IFI16-interacting proteins, such as p53 and BRCA1, would not have necessarily implied that these proteins participate with IFI16 in the recognition of HSV-1 genomes and vice versa. To examine that issue would require the use of immunofluorescence assays to observe any possible colocalization among the proteins of interest and the incoming viral DNA, or chromatin immunoprecipitation assays. Indeed, it has recently been reported that BRCA1 regulates the herpesviral DNA nuclear sensing by IFI16 and the induction of inflammasome and type I IFN responses. These authors reported that BRCA1 is complexed with IFI16 in the nucleus of host cells, and after herpesviral genome recognition BRCA1 translocates into the cytosol together with the IFI16 inflammasome (Dutta, Dutta et al. 2015). This observation is similar to the movement of BRCA1 to the cytosol after DNA damage in a p53-dependent manner (Feng, Kachnic et al. 2004; Jiang, Yang et al. 2011), indicating once more the analogy between the responses against DNA damage and viral infections.

As described in the introduction, HSV-1 has evolved to regulate components of the same pathway differentially to promote its own replication. For example, HSV-1 infection results in the activation of ATM and downstream targets including Chk2 and p53 (Lilley, Carson et al. 2005; Shirata, Kudoh et al. 2005; Li, Baskaran et al. 2008), however ICP0 prevents full activation of the ATM-dependent pathway by inducing the degradation of RNF8 and RNF168, which are key mediators in this signalling pathway (Lilley, Chaurushiya et al. 2010). Given that BRCA1 is an important mediator in the ATM-signalling pathway and the 120 KDa-band was reactive with an antibody for BRCA1, the upregulation of the 120 kDa protein was intriguing. Since this band could be a potential splice variant of BRCA1, a product of BRCA1 cleavage induced by HSV-1 or another protein, a detailed characterization of this 120 KDa protein was undertaken.

From these data it can be concluded that the upregulation of the 120 KDa-protein was specific to HSV-1 infection because, at least VACV, HCMV and HSV-2 infections did not alter the levels of this protein (Figs. R10, R11 and R14).

Upregulation of the 120 KDa-protein during HSV-1 infection was also observed in all the cell

lines analyzed, independently of their origin (Figs. R9, R10 and R36).

Furthermore, the 120 KDa-protein was up-regulated during the infection with all the HSV-1 mutants used, even during the infection with replication-defective mutant viruses; therefore, productive viral replication was not necessary to stimulate the up-regulation of the 120 KDa-protein, although infection with the mutants with a more restrictive phenotype (tsK and *in*1382) was associated with lower levels of induction of the protein (Figs. R13 and R14).

It is important to note that in some immunoblot analyses the antibody I-20 recognized another protein of around 80 KDa in lysates of HSV-1 infected cells. Moreover, when lysates of uninfected cells were analysed another band appeared, but its molecular weight was a bit higher than the 120 KDa band observed on infected cells.

Since a band of approximately 220-230 kDa corresponding to full-length BRCA1 was not observed in our experiments with the I-20 antibody, another antibody against BRCA1 was used to assess the up-regulation of the putative BRCA1 related 120 kDa-protein during HSV-1 infection, and discard any possible I-20 antibody crossreactivity. Given that the I-20 antibody was raised by immunization with a peptide of the C-terminal region of the human BRCA1, the D-20 antibody (Santa Cruz Biotechnology) that was raised by immunization with a peptide from the N-terminus of the molecule was used. However, this antibody showed extensive cross-reactivity and poor specificity for BRCA1 making interpretation of these data problematic. The D-20 antibody recognized mainly a protein of 80-90 kDa in HSV-1 infected cells, but it did not recognize the 120 kDa protein or full-length BRCA1 (Figs. R15, R16 and R17).

Different proteins were detected by the I-20 and D-20 antibodies, and a protein that can be unequivocally identified as full-length BRCA1 was not recognized by these antibodies, as other authors stated previously (Wilson 1996). Moreover, the difficulty to detect endogenous full-length BRCA1 by different techniques including western blot analysis is widely known (Thomas, Smith et al. 1996; Wilson, Payton et al. 1996; Bernard-Gallon, Crespin et al. 1997; Wilson, Payton et al. 1997; Imakado, Hoashi et al. 1998; Wilson, Ramos et al. 1999; Yoshikawa, Honda et al. 1999; Perez-Valles, Martorell-Cebollada et al. 2001; Bogdani, Teugels et al. 2002; Milner, Wombwell et al. 2013). Thus, the presence of the bands observed not at the proper molecular weight could represent splice variants, cleavage products, or cross-reactive binding to non BRCA1 proteins. These concerns prompted us to investigate the identity of the 120 kDa protein more deeply.

An initial hypothesis to explain the observation of bands of 120 kDa and 80-90 kDa instead of full-length BRCA1 (220-230 kDa) was a possible cleavage of BRCA1 induced by HSV-1 infection. Proteolysis of the full-length molecule might render the epitopes of the I-20 and D-20 antibodies more “accessible”; therefore, the N-terminus fragment around 90 KDa could be recognized by the D-20 antibody and the C-terminal fragment around 120 KDa could be recognized by the I-20 antibody. As

well as I-20 and D-20 antibodies, we used the N-terminal MS110 antibody to study our hypothesis. However, our immunoprecipitation-immunoblot and immunofluorescence analyses of U2OS stably expressing full-length BRCA1 suggested that the full-length molecule was not cleaved during HSV-1 infection (Figs. R28, R29 and R34).

One issue that complicated all these analyses was the lack of consistency between the antibodies used. This has been a general problem in studies of the cell biology of BRCA1; indeed there has been much controversy with regard to the size and localization of BRCA1 protein explained largely by differences in the specificity of the antibodies used in the various studies. The specificity of several BRCA1 antibodies has been extensively debated previously and this issue is exemplified by the crossreactivity of the C-terminal C-20 antibody (Santa Cruz Biotechnology) with EGFR. Initial reports using the C-20 antibody identified BRCA1 as approximately a 190 kda protein which functioned as a secreted growth inhibitor (Gudas, Nguyen et al. 1995; Gudas, Li et al. 1996; Holt, Thompson et al. 1996; Jensen, Thompson et al. 1996). However, subsequent studies revealed that the band of 190 kDa recognized by this antibody corresponded to EGFR. This antibody was also found to crossreact with HER2, another member of the type I tyrosine kinase receptor family, and with as yet undefined antigen expressed by smooth muscle cells (Thomas, Smith et al. 1996; Wilson, Payton et al. 1996; Bernard-Gallon, Crespin et al. 1997; Imakado, Hoashi et al. 1998).

The I-20 antibody is also a C-terminal antibody but the epitope is adjacent to that of the C-20 antibody. Although other published data (Wilson 1996) and our own work (Fig. R18) showed that the I-20 antibody does not crossreact with EGFR, the specificity of this antibody has been questioned in several publications (Wilson, Payton et al. 1996; Wilson, Payton et al. 1997; Wilson, Ramos et al. 1999; Yoshikawa, Honda et al. 1999; Perez-Valles, Martorell-Cebollada et al. 2001; Bogdani, Teugels et al. 2002). In particular, it was found that this antibody recognized other proteins different from BRCA1 in different cells including the cell line HCC1937, even though these cells express a truncated BRCA1 protein which lacks the C-terminus of BRCA1. It has been argued that these additional proteins were unlikely to be related to BRCA1 since they were not detected with other BRCA1 antibodies (Wilson 1997, 1999, Yoshikawa 1999).

Thus, although the I-20 antibody clearly recognized overexpressed transfected GFP-BRCA1 (Figs. R27-R29 and R34), it may also bind cross-reactively to other antigens. To definitively resolve the identity of the 120 kDa protein up-regulated after HSV-1 infection we used immunoprecipitation from HSV-1-infected Vero cells using the I-20 antibody followed by mass spectrometry analysis to obtain the amino acid sequence of the immunoprecipitated proteins, especially the 120 kDa protein (Figs. R36 and R37).

These mass spectrometry data clearly identified **the I-20 reactive band at 120 kDa as the HSV-1 tegument protein UL37** (Fig. R38), which has a predicted molecular weight of 120 kDa. Interestingly, bands at 150 and 80 kDa which were also subjected to mass spectrometry analysis were also identified as viral proteins, the major capsid protein and the tegument protein VP13/VP14 respectively.

To further confirm the result about the 120 kDa protein experiments involving immunoprecipitation using the I-20 antibody followed by western blot analysis with an antibody specific for UL37 were performed. A clear signal at 120 kDa for this antibody was observed, which indicated that I-20 is able to bind UL37 (Fig. R39-A). In further confirmatory experiments, the I-20 antibody was unable to detect any 120 kDa protein when cells were infected with a virus deficient in UL37 (Fig. R39-B). Using different constructs expressing either only the N-terminus of UL37 or lacking this part of the molecule (Fig. R39-C), we confirmed that I-20 binds specifically to the N-terminus region of UL37 as predicted from the alignment between the peptide used to generate the antibody I-20 and the UL37 protein. This alignment revealed six identical amino acids between the N-terminus region of UL37 and the C-terminus of BRCA1 (BRCT2 domain) (Fig. R38). Interestingly, the published crossreactivity of the C-20 antibody with EGFR was also due to a similarity between the C-20 epitope and EGFR consisting of six identical aminoacids as well as three conservative substitutions (Wilson 1996).

In spite of the similarity between HSV-1 and HSV-2, the alignment between UL37 proteins from HSV-1 and HSV-2 revealed that the six aminoacids identical between the I-20 epitope and HSV-1 UL37 were not conserved in UL37 from HSV-2, explaining why the 120 kDa protein was not detected in HSV-2 infected cells.

It is of additional interest that immunoblotting with I-20 on total protein extracts of uninfected cells give rise to a band of 130 kDa approximately, as mentioned before. While it is possible that this band represents a BRCA1 species, the fact that it was mainly detected under less restrictive conditions and full length BRCA1 cannot be detected suggests that this band represent and additional protein different from BRCA1. A BLAST alignment between the I-20 peptide and the human data base revealed PAXIP1 (PAX-interacting protein 1) as a possible candidate. This molecule has a molecular weight of 130 kDa and the I-20 epitope aligned to the BRCT2 domain of PAXIP1. However, further investigation would be needed to clarify this issue.

We have not observed a 220-230 Kda band representing full-length endogenous BRCA1 using the commercial antibodies I-20 and D-20 in immunoblots of total cell proteins. However, consistent with previous published reports, the commercially available antibodies used in our study (I-20, D-20 and MS110) could identify overexpressed BRCA1 protein in immunoprecipitation and/or immunofluorescence analyses (Figs. R27-R30 and R34). Thus, the difficulties in detection of the endogenously expressed molecule could indicate that the abundance of this molecule is limited post-translationally as previously proposed (Wilson, Payton, Oncogene 1997).

In the field of breast cancer research, the fact that many BRCA1 antibodies crossreact with other cellular proteins has given rise to much scepticism about BRCA1 data at protein level. Here, we reported the first crossreaction of a BRCA1 antibody with a viral protein, and given the track record of BRCA1 antibodies and cross-reactivities these data reinforce the need for careful validation of antibodies used in experiments studying this molecule. In particular, some of the antibodies used in this thesis have also been used by other groups for example studying the changes in BRCA1 distribution during HSV-1 infection. This does not mean that those data are erroneous; simply that great caution

should be taken when interpreting past and future results generated using the I-20 antibody in HSV-1 infected cells.

As described in the introduction, IFI16 protein is associated with cell growth inhibition, cell cycle arrest and cellular senescence. The function of IFI16 in the regulation of cell proliferation depends on its ability to bind other proteins and modulate their activities. Up to now, a number of proteins are known to interact with IFI16, including Rb and E2F, BRCA1 and p53. In part, IFI16 could inhibit cell proliferation by upregulating p21 expression in p53-dependent and independent manner and potentiating the Rb/E2F-stimulated transcriptional inhibition (Xin, Curry et al. 2003; Xin, Pereira-Smith et al. 2004). Although a variety of evidences indicated that the interaction of IFI16 with p53 enhanced p53 transcriptional activity (Johnstone, Wei et al. 2000; Fujiuchi, Aglipay et al. 2004; Xin, Pereira-Smith et al. 2004), it is not yet clear whether p53 in turn could regulate IFI16 expression or activity. Since a number of proteins that regulate p53 functions are in turn regulated by p53 such as p202 (Datta, Li et al. 1996; D'Souza, Xin et al. 2001), it seemed reasonable to test whether p53 could regulate the expression or activity of IFI16. We have observed that the p53 knockout HCT116 cells barely express IFI16 compared to the wt HCT116 cells. The reduced expression of IFI16 in HCT116 $p53^{-/-}$ cells could be associated with the lack of p53 if p53 regulates IFI16 expression, at either a transcriptional or translational level, or IFI16 stability. Given that IFI16 is localized mainly in the nucleus of HCT116 cells, any regulation of IFI16 by p53 might be more likely to occur at a transcriptional level. Moreover, given that p53 is a well known transcriptional regulator, it seemed plausible that p53 regulated IFI16 at transcriptional level, although it is known that p53 acts as more than just a transcription factor (Caelles, Helmberg et al. 1994; Haupt, Rowan et al. 1995; Ding, Lin et al. 2000; Chipuk, Maurer et al. 2003; Chipuk, Kuwana et al. 2004). Furthermore, two key characteristics of the p53 protein are required for its capacity to promote or inhibit transcription of a variety of genes: its ability to recognize and bind specific DNA sequences and to recruit components of the transcriptional machinery or transcriptional co-regulators. Therefore, if p53 is a DNA sequence-specific transcriptional regulator, and IFI16 was transcriptionally regulated by p53, IFI16 should have p53 DNA-binding sites. However, there is only one report that has identified a putative p53 DNA-binding site within the first exon of the *IFI16* gene (Song 2008).

If the marked reduction of IFI16 observed in the p53 knockout HCT116 cells was associated with the lack of p53, we wondered if the reconstitution of p53 expression could restore IFI16 expression. Since no re-expression of IFI16 protein was observed after p53 transfection of HCT116 p53-null cells, we analysed whether the activation of p53 was required to promote the transcription of *IFI16* gene. Moreover, the single report about IFI16 up-regulation by p53 showed that the transcription of the *IFI16* gene was stimulated in response to the functional activation of p53. Therefore, after p53 transfection we treated the cells with two different DNA-damaging agents: short wave ultraviolet radiation (254nm) as a non-ionizing radiation (Fig. R44) or gamma radiation (Cesium 137,10Gy) as an ionizing radiation (Fig. R43). Although these results suggested that p53 was not able to induce the

up-regulation of IFI16 expression after exposure to ionizing and non-ionizing radiation, the absence of a control for p53 activation, for example analysis of the phosphorylation of p53 on Ser15, renders difficult a confident interpretation of these data. An additional positive control to verify the activation of p53 would have been to analyse the levels of known transcriptional targets of p53, such as p21.

A complementary approach to check if activated p53 could stimulate IFI16 expression would have been to treat the wt HCT116 cells with an inhibitor of p53-mediated functions or to silence p53 expression and observe the effects of these treatments on IFI16 expression levels before and after treatment with DNA-damaging agents.

Further planned experiments included verification of the integrity of *IFI16* gene in the genomic DNA of the HCT116 p53-null cells as a necessary validation before proceeding to assay the possible p53 transcriptional regulation of IFI16 by comparing the IFI16 mRNA levels of wt and p53-null HCT116 cells by quantitative real-time PCR (qRT-PCR).

Interestingly, a study carried out by other authors, where they assessed whether the p53-dependent enhancement of IFN signalling and production was mediated by p53 transcriptional up-regulation of IRF9, revealed that the tranfection of IRF9 into HCT116 $p53^{-/-}$ cells significantly increased the transactivation of an ISRE reporter but it could not completely restore the levels achieved in HCT116 expressing endogenous p53. The authors suggested that p53 might have, in addition to IRF9, other IFN-related target genes (Munoz-Fontela, Macip et al. 2008). Since we have observed that p53-null HCT116 cells barely express IFI16 compared to the wt cells, we speculated that IFI16 could be an additional transcriptional target of p53 that might contribute to the enhancement of IFN signalling mediated by p53. However, our hypothesis remains at present speculative.

Nevertheless, these experiments had to be interrupted because both wt and p53 knockout HCT116 cells started to grow as spheroids instead of as a monolayer. When the wt cells formed these spheroids they expressed markedly less IFI16 than usual. Intriguingly, no other batches of wt HCT116 cells supplied by other groups expressed IFI16 protein. Therefore, it proved impossible to continue experiments with these cell lines.

While the above experiments were in progress the study of IFI16 expression was extended to include other cells of known p53 status. Since it is known that levels of IFI16 mRNA and protein are frequently decreased in many breast cancer cell lines (Fujiuchi 2004), a possible relation between IFI16 expression and p53 status was analyzed in several breast cell lines. However, we did not observe an obvious relation between the expression of wt p53 and IFI16 in the breast cell lines analyzed (Fig. R45). This could indicate that there are more factors involved in IFI16 regulation than just p53. For instance, the MCF7 cell line expresses wt p53 (although the molecule ran slightly slower than the p53 protein from the normal MCF10A cell line) but they did not express IFI16 (Fig. R45 A-B). Since the

MCF7 cell line line is tumoral, it is possible that it expresses wt p53 but has mutated or does not express other proteins required for normal IFI16 expression. Moreover, although the majority of the breast cancer cell lines that have mutant p53 showed no IFI16 expression, the Hs578T cell line expressed IFI16 levels comparable to MCF10A and the BT-549 cell line expressed low levels of IFI16 (Fig. R45 A,B and C). Although both cell lines have a missense mutation in the *p53* sequence, it could be possible that these mutations do not affect significantly to the structure or function of p53 protein comparing to the p53 missense mutations in the other cell lines that do not express IFI16. However, according to the data base from T.Soussi the Hs578T cells preserve only 9,06% of p53 functionality (as determined by the residual transcriptional activity of mutant p53 on the p21 promoter compared to wt p53) and the BT-549 cells maintain 12,42% activity. Both p53 activities are higher than in other cell lines that do not express IFI16, but they are also similar to the p53 functionality of other cell lines that do not express IFI16. To clarify this issue more studies would be needed in order to determine the molecular factors that regulate IFI16 expression, and then compare them among the different breast cancer cell lines. It should be expected that the cancer cell lines that do not express IFI16 have mutations in these factors regulating IFI16 expression in addition to p53. And maybe in the BT-549 and Hs578T cell lines these factors are unaffected or less affected and for this reason the IFI16 expression is stably or slightly maintained in the Hs578T and BT-549 cells, respectively. Moreover, in both BT-549 and Hs578T cell lines there could be a mutation in *IFI16* gene that increased or maintained its levels of expression in spite of the process of tumoral transformation.

Although the BT-549 cell line expressed IFI16, its levels were considerably reduced compared to those of the normal MCF10A cell line. Thus, except the Hs578T cell line, all the breast cancer cell lines that have mutant p53 are associated with a loss or reduced expression of IFI16. These results were consistent with previous findings that indicate a frequent loss of IFI16 expression in breast cancer cells (Fujiuchi, Aglipay et al. 2004), thereby supporting the notion that IFI16 is involved in relevant tumour suppressor functions in breast cancer. To further analyse the loss of IFI16 function in breast cancer development, it would have been interesting to check the expression of IFI16 in a normal mammary cell line, for example MCF10A, to test whether IFI16 expression decreases after or during the transformation of the mammary epithelium.

To study if a reduction of IFI16 expression was a consistent feature of tumour cells, a panel of bladder tumour cell lines were also analysed for IFI16 expression. All the bladder cell lines analyzed expressed IFI16. Moreover, the p53 status of these bladder tumour cell lines did not correlate with the IFI16 levels, since the RT4 cell line that contains wt p53 is the one which expressed the lowest levels of IFI16.

One interpretation of these results might be that the regulation of IFI16 expression by p53 could be tissue-dependent, which in turn would indicate that the putative oncosuppressive function of IFI16 could be dependent on the cellular type. In agreement with this idea, although previous observations have supported the idea that the loss of function of IFI16 may provide growth advantage to the affected cells, co-expression of AIM2 and IFI16 enhanced the cell growth of an oral squamous

cell carcinoma cell line deficient for p53. This suggested that expression of AIM2 and IFI16 may have oncogenic function in this type of carcinoma that lacks p53 (Kondo, Nagai et al. 2012). Interestingly, the expression of both proteins after introducing wt p53 suppressed cell growth. Moreover, this could indicate that wt p53 might regulate the activity of IFI16 in these cells.

The fact that IFI16 has an oncosuppressive function mainly in breast and prostate cells, and BRCA1 is a critical tumour suppressor protein involved in the development of many types of breast and prostate cancer, suggest that the role of IFI16 in tumour suppressor activities in these type of tumours might be related to the capacity of IFI16 to interact with BRCA1. Moreover, the biological function of IFI16 in breast and prostate cancer development might also be related to the interaction of IFI16 with the glucocorticoid receptor (GR) and the androgen receptor (AR). GR and AR are steroid hormone receptors that play important roles in the development of breast and prostate cancers (reviewed in (Kach, Conzen et al. 2015)). Since IFI16 has been considered as a modulator of glucocorticoid and androgen actions by binding to GR and AR respectively (Alimirah, Chen et al. 2006; Berry, Matthews et al. 2010), maybe the loss of IFI16 function in these cells could lead to deregulated GR and AR activities promoting breast and prostate cancer.

One of the hallmarks of human malignancies is their genomic instability due to compromised DDR pathways. The fact that viral proteins such as ICP0 can disrupt the DDR by degrading key cellular proteins involved in DNA damage sensing, signaling and/or repair, including IFI16, may contribute to genome instability and hence human cancer development. Thus, clarification of mechanisms underlying the interactions between virus and DDR pathways would be a fundamental area of study.

Conclusions

1. Infection with HSV-1 leads to a marked reduction in IFI16 expression levels. IFI16 protein levels are slightly reduced after infection with an ICP0 deficient virus, but the reduction in IFI16 expression levels is markedly enhanced if the HSV-1 expresses ICP0, although the loss of IFI16 varies to some extent between different cell lines.

2. Analysis of mutant viruses that express ICP0 but not additional IE gene products showed that loss of IFI16 occurs even in the absence of efficient viral replication.

3. Analysis of the expression of p53 after HSV-1 infection showed that p53 expression levels were not consistently affected in the cell lines analysed.

4. Analysis of the expression of BRCA1 after HSV-1 infection using the commercially available BRCA1 antibody I-20 (Santa Cruz Biotechnology) revealed a marked increased in a 120 kDa protein. This phenotype was observed in all the cell lines analysed and was specific to HSV-1 infection since this band was not observed after infection with other viruses.

5. The proteomic analysis revealed that the 120 kDa-band up-regulated in HSV-1 infected cells observed with the I-20 antibody was UL37, an HSV-1 tegument protein. Thus, a BRCA1 antibody cross-reacts with the HSV-1 UL37 protein. The cross-reactive epitope was localised in the N-terminal region of UL37 in transfection studies with plasmids expressing different fragments of UL37.

6. The expression of IFI16 proteins is markedly reduced in the p53 knockout HCT116 colorectal carcinoma cell line compared to the levels expressed by wild type HCT116 cells. Reconstitution of the expression of wt p53 had no appreciable effect on IFI16 expression. Furthermore, transfected p53 did not affect the expression levels of endogenous and transfected IFI16 after exposure to either ultraviolet or gamma radiation.

7. Analysis of the expression of IFI16 protein in different breast cell lines showed that the majority of the breast cancer cell lines analysed have lost or reduced expression of IFI16. However, a clear relation between IFI16 expression and p53 status was not observed. Moreover, analysis of IFI16 expression in a panel of bladder tumour cell lines showed that all these cells expressed IFI16 and IFI16 expression levels did not correlate with p53 status.

1. La infección por HSV-1 desencadena una marcada reducción en los niveles de expresión de IFI16. Los niveles de proteína de IFI16 disminuyen ligeramente tras la infección por un virus deficiente en ICP0, sin embargo esta reducción en los niveles de expresión de IFI16 se potencia notablemente si el virus expresa ICP0, aunque el grado de pérdida de IFI16 varía entre diferentes líneas celulares.

2. El análisis mediante virus mutantes que expresan ICP0 pero carecen de otros genes temprano-inmediatos indica que la reducción de los niveles de IFI16 puede ocurrir incluso en ausencia de una replicación viral eficiente.

3. El estudio de la expresión de p53 tras la infección por HSV-1 muestra que los niveles de esta proteína no varían de manera consistente en las líneas celulares estudiadas.

4. El análisis de la expresión de BRCA1 tras la infección por HSV-1 utilizando el anticuerpo comercial I-20 (Santa Cruz Biotechnology) revela un marcado aumento de una proteína de 120 kDa. Este fenotipo se manifiesta en todas las líneas celulares analizadas y es específico de infección por HSV-1, ya que no se observa tras la infección con ninguno de los otros virus utilizados.

5. El análisis de proteómica revela que la banda de 120 kDa que aumenta en células infectadas por HSV-1 cuando se utiliza el anticuerpo I-20 es UL37, una proteína del tegumento del HSV-1. Por lo tanto I-20, un anticuerpo contra BRCA1, reconoce la proteína de HSV-1 UL37. El epítopo que produce esta reactividad cruzada se localiza en la región N-terminal de UL37, como se determinó en estudios de transfección con plásmidos que expresan diferentes fragmentos de UL37.

6. La expresión de la proteína IFI16 se reduce marcadamente en la línea celular de carcinoma colorrectal HCT116 deficiente en p53, en comparación a los niveles de las células HCT116 control. La reconstitución de la expresión de p53 no supone un efecto apreciable en la expresión de IFI16. Además, p53 transfectado tampoco afecta a los niveles de expresión de IFI16, endógeno o transfectado, tras la exposición a radiación ultravioleta o gamma.

7. El análisis de la expresión de la proteína IFI16 en diferentes líneas celulares de mama muestra que la mayoría de las líneas celulares de cáncer de mama analizadas presentan una pérdida o reducción notable de los niveles de expresión de IFI16. Sin embargo, no se observa una clara relación entre los niveles de IFI16 y el estado de p53. Además, el análisis de la expresión de IFI16 en un panel de líneas celulares procedentes de tumores de vejiga, muestra que todas estas células expresan IFI16 y sus niveles de expresión no correlacionan con el estado de p53.

Bibliography

Abe, T., A. Harashima, et al. (2013). "STING recognition of cytoplasmic DNA instigates cellular defense." Mol Cell 50(1): 5-15.

Ablasser, A., F. Bauernfeind, et al. (2009). "RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate." Nat Immunol 10(10): 1065-72.

Ace, C. I., M. A. Dalrymple, et al. (1988). "Mutational analysis of the herpes simplex virus type 1 trans-inducing factor Vmw65." J Gen Virol 69 (Pt 10): 2595-605.

Ace, C. I., T. A. McKee, et al. (1989). "Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression." J Virol 63(5): 2260-9.

Aglipay, J. A., S. W. Lee, et al. (2003). "A member of the Pyrin family, IFI16, is a novel BRCA1-associated protein involved in the p53-mediated apoptosis pathway." Oncogene 22(55): 8931-8.

Al-Mulla, F., M. Abdulrahman, et al. (2005). "BRCA1 gene expression in breast cancer: a correlative study between real-time RT-PCR and immunohistochemistry." J Histochem Cytochem 53(5): 621-9.

Alamshah, A., R. Springall, et al. (2008). "Use of a BRCA1 peptide validates MS110 as a BRCA1-specific antibody in immunohistochemistry." Histopathology 53(1): 117-20.

Albrecht, M., D. Choubey, et al. (2005). "The HIN domain of IFI-200 proteins consists of two OB folds." Biochem Biophys Res Commun 327(3): 679-87.

Alimirah, F., J. Chen, et al. (2006). "Androgen receptor auto-regulates its expression by a negative feedback loop through upregulation of IFI16 protein." FEBS Lett 580(6): 1659-64.

Amici, C., A. Rossi, et al. (2006). "Herpes simplex virus disrupts NF-kappaB regulation by blocking its recruitment on the IkappaBalpha promoter and directing the factor on viral genes." J Biol Chem 281(11): 7110-7.

Ansari, M. A., V. V. Singh, et al. (2013). "Constitutive interferon-inducible protein 16-inflammasome activation during Epstein-Barr virus latency I, II, and III in B and epithelial cells." J Virol 87(15): 8606-23.

Barbe, F., T. Douglas, et al. (2014). "Advances in Nod-like receptors (NLR) biology." Cytokine Growth Factor Rev 25(6): 681-97.

Barker, J. R., B. J. Koestler, et al. (2013). "STING-dependent recognition of cyclic di-AMP mediates type I interferon responses during Chlamydia trachomatis infection." MBio 4(3): e00018-13.

Bartek, J., J. Bartkova, et al. (1991). "Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies." Oncogene 6(9): 1699-703.

Bartek, J., J. Bartkova, et al. (1990). "Patterns of expression of the p53 tumour suppressor in human breast tissues and tumours in situ and in vitro." Int J Cancer 46(5): 839-44.

Bartek, J., R. Iggo, et al. (1990). "Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines." Oncogene 5(6): 893-9.

Bau, D. T., Y. P. Fu, et al. (2004). "Breast cancer risk and the DNA double-strand break end-joining capacity of nonhomologous end-joining genes are affected by BRCA1." Cancer Res 64(14): 5013-9.

Baucke, R. B. and P. G. Spear (1979). "Membrane proteins specified by herpes simplex viruses. V. Identification of an Fc-binding glycoprotein." J Virol 32(3): 779-89.

Bell, S., M. Cranage, et al. (1990). "Induction of immunoglobulin G Fc receptors by recombinant vaccinia viruses expressing glycoproteins E and I of herpes simplex virus type 1." J Virol 64(5): 2181-6.

Bernard-Gallon, D. J., N. C. Crespin, et al. (1997). "Cross-reaction between antibodies raised against the last 20 C-terminal amino acids of BRCA 1 (C-20) and human EGF and EGF-R in MCF 10a human mammary epithelial cell line." Int J Cancer 71(1): 123-6.

Berry, A., L. Matthews, et al. (2010). "Interferon-inducible factor 16 is a novel modulator of glucocorticoid action." FASEB J 24(6): 1700-13.

Beswick, T. S. (1962). "The origin and the use of the word herpes." Med Hist 6: 214-32.

Bieniasz, P. D. (2004). "Intrinsic immunity: a front-line defense against viral attack." Nat Immunol 5(11): 1109-15.

Bochar, D. A., L. Wang, et al. (2000). "BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer." Cell 102(2): 257-65.

Bogdani, M., E. Teugels, et al. (2002). "Loss of nuclear BRCA1 localization in breast carcinoma is age dependent." Virchows Arch 440(3): 274-9.

Boutell, C., M. Canning, et al. (2005). "Reciprocal activities between herpes simplex virus type 1 regulatory protein ICP0, a ubiquitin E3 ligase, and ubiquitin-specific protease USP7." J Virol 79(19): 12342-54.

Boutell, C. and R. D. Everett (2003). "The herpes simplex virus type 1 (HSV-1) regulatory protein ICP0 interacts with and Ubiquitinates p53." J Biol Chem 278(38): 36596-602.

Boutell, C. and R. D. Everett (2004). "Herpes simplex virus type 1 infection induces the stabilization of p53 in a USP7- and ATM-independent manner." J Virol 78(15): 8068-77.

Boutell, C. and R. D. Everett (2013). "Regulation of alphaherpesvirus infections by the ICP0 family of proteins." J Gen Virol 94(Pt 3): 465-81.

Brazda, V., J. Coufal, et al. (2012). "Preferential binding of IFI16 protein to cruciform structure and superhelical DNA." Biochem Biophys Res Commun 422(4): 716-20.

Briggs, L. J., R. W. Johnstone, et al. (2001). "Novel properties of the protein kinase CK2-site-regulated nuclear- localization sequence of the interferon-induced nuclear factor IFI 16." Biochem J 353(Pt 1): 69-77.

Brosh, R. and V. Rotter (2009). "When mutants gain new powers: news from the mutant p53 field." Nat Rev Cancer 9(10): 701-13.

Brunette, R. L., J. M. Young, et al. (2012). "Extensive evolutionary and functional diversity among mammalian AIM2-like receptors." J Exp Med 209(11): 1969-83.

Brzostek-Racine, S., C. Gordon, et al. (2011). "The DNA damage response induces IFN." J Immunol 187(10): 5336-45.

Bunz, F., A. Dutriaux, et al. (1998). "Requirement for p53 and p21 to sustain G2 arrest after DNA damage." Science 282(5393): 1497-501.

Burckstummer, T., C. Baumann, et al. (2009). "An orthogonal proteomic-genomic screen identifies

AIM2 as a cytoplasmic DNA sensor for the inflammasome." Nat Immunol 10(3): 266-72.
Burdette, D. L., K. M. Monroe, et al. (2011). "STING is a direct innate immune sensor of cyclic di-GMP." Nature 478(7370): 515-8.
Burrus, G. R., J. A. Briggs, et al. (1992). "Characterization of the human myeloid cell nuclear differentiation antigen: relationship to interferon-inducible proteins." J Cell Biochem 48(2): 190-202.
Caelles, C., A. Helmberg, et al. (1994). "p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes." Nature 370(6486): 220-3.
Campbell, M. E., J. W. Palfreyman, et al. (1984). "Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription." J Mol Biol 180(1): 1-19.
Caposio, P., F. Gugliesi, et al. (2007). "A novel role of the interferon-inducible protein IFI16 as inducer of proinflammatory molecules in endothelial cells." J Biol Chem 282(46): 33515-29.
Carpenter, G., K. J. Lembach, et al. (1975). "Characterization of the binding of 125-I-labeled epidermal growth factor to human fibroblasts." J Biol Chem 250(11): 4297-304.
Chelbi-Alix, M. K. and H. de The (1999). "Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins." Oncogene 18(4): 935-41.
Chen, J., D. P. Silver, et al. (1998). "Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells." Mol Cell 2(3): 317-28.
Chipuk, J. E., T. Kuwana, et al. (2004). "Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis." Science 303(5660): 1010-4.
Chipuk, J. E., U. Maurer, et al. (2003). "Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription." Cancer Cell 4(5): 371-81.
Chisholm, S. E., K. Howard, et al. (2007). "Expression of ICP0 is sufficient to trigger natural killer cell recognition of herpes simplex virus-infected cells by natural cytotoxicity receptors." J Infect Dis 195(8): 1160-8.
Chiu, Y. H., J. B. Macmillan, et al. (2009). "RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway." Cell 138(3): 576-91.
Choubey, D. and J. U. Gutterman (1997). "Inhibition of E2F-4/DP-1-stimulated transcription by p202." Oncogene 15(3): 291-301.
Choubey, D. and P. Lengyel (1995). "Binding of an interferon-inducible protein (p202) to the retinoblastoma protein." J Biol Chem 270(11): 6134-40.
Choubey, D., S. J. Li, et al. (1996). "Inhibition of E2F-mediated transcription by p202." EMBO J 15(20): 5668-78.
Choubey, D., J. Snoddy, et al. (1989). "Interferons as gene activators. Indications for repeated gene duplication during the evolution of a cluster of interferon-activatable genes on murine chromosome 1." J Biol Chem 264(29): 17182-9.
Cliffe, A. R. and D. M. Knipe (2008). "Herpes simplex virus ICP0 promotes both histone removal and acetylation on viral DNA during lytic infection." J Virol 82(24): 12030-8.

Costa, S., C. Borgogna, et al. (2011). "Redistribution of the nuclear protein IFI16 into the cytoplasm of ultraviolet B-exposed keratinocytes as a mechanism of autoantigen processing." Br J Dermatol 164(2): 282-90.

Cridland, J. A., E. Z. Curley, et al. (2012). "The mammalian PYHIN gene family: phylogeny, evolution and expression." BMC Evol Biol 12: 140.

Cristea, I. M., N. J. Moorman, et al. (2010). "Human cytomegalovirus pUL83 stimulates activity of the viral immediate-early promoter through its interaction with the cellular IFI16 protein." J Virol 84(15): 7803-14.

Cuchet-Lourenco, D., G. Anderson, et al. (2013). "The viral ubiquitin ligase ICP0 is neither sufficient nor necessary for degradation of the cellular DNA sensor IFI16 during herpes simplex virus 1 infection." J Virol 87(24): 13422-32.

Cuchet-Lourenco, D., C. Boutell, et al. (2011). "SUMO pathway dependent recruitment of cellular repressors to herpes simplex virus type 1 genomes." PLoS Pathog 7(7): e1002123.

D'Souza, S., H. Xin, et al. (2001). "The gene encoding p202, an interferon-inducible negative regulator of the p53 tumor suppressor, is a target of p53-mediated transcriptional repression." J Biol Chem 276(1): 298-305.

Datta, B., B. Li, et al. (1996). "p202, an interferon-inducible modulator of transcription, inhibits transcriptional activation by the p53 tumor suppressor protein, and a segment from the p53-binding protein 1 that binds to p202 overcomes this inhibition." J Biol Chem 271(44): 27544-55.

Davidoff, A. M., P. A. Humphrey, et al. (1991). "Genetic basis for p53 overexpression in human breast cancer." Proc Natl Acad Sci U S A 88(11): 5006-10.

Davidson, N. E., E. P. Gelmann, et al. (1987). "Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines." Mol Endocrinol 1(3): 216-23.

Davison, M. J., V. G. Preston, et al. (1984). "Determination of the sequence alteration in the DNA of the herpes simplex virus type 1 temperature-sensitive mutant ts K." J Gen Virol 65 (Pt 5): 859-63.

Dawson, M. J., N. J. Elwood, et al. (1998). "The IFN-inducible nucleoprotein IFI 16 is expressed in cells of the monocyte lineage, but is rapidly and markedly down-regulated in other myeloid precursor populations." J Leukoc Biol 64(4): 546-54.

Dawson, M. J. and J. A. Trapani (1995a). "IFI 16 gene encodes a nuclear protein whose expression is induced by interferons in human myeloid leukaemia cell lines." J Cell Biochem 57(1): 39-51.

Dawson, M. J. and J. A. Trapani (1995b). "The interferon-inducible autoantigen, IFI 16: localization to the nucleolus and identification of a DNA-binding domain." Biochem Biophys Res Commun 214(1): 152-62.

DeLuca, N. A., A. M. McCarthy, et al. (1985). "Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4." J Virol 56(2): 558-70.

Demaison, C., K. Parsley, et al. (2002). "High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based

lentiviral vector containing an internal spleen focus forming virus promoter." Hum Gene Ther 13(7): 803-13.

Deschamps, S., J. Meyer, et al. (2003). "The mouse Ifi200 gene cluster: genomic sequence, analysis, and comparison with the human HIN-200 gene cluster." Genomics 82(1): 34-46.

Deshaies, R. J. and C. A. Joazeiro (2009). "RING domain E3 ubiquitin ligases." Annu Rev Biochem 78: 399-434.

Desmyter, J., J. L. Melnick, et al. (1968). "Defectiveness of interferon production and of rubella virus interference in a line of African green monkey kidney cells (Vero)." J Virol 2(10): 955-61.

DeYoung, K. L., M. E. Ray, et al. (1997). "Cloning a novel member of the human interferon-inducible gene family associated with control of tumorigenicity in a model of human melanoma." Oncogene 15(4): 453-7.

Diao, L., B. Zhang, et al. (2005). "Herpes virus proteins ICP0 and BICP0 can activate NF-kappaB by catalyzing IkappaBalpha ubiquitination." Cell Signal 17(2): 217-29.

Diefenbach, R. J., M. Miranda-Saksena, et al. (2008). "Transport and egress of herpes simplex virus in neurons." Rev Med Virol 18(1): 35-51.

Diner, B. A., T. Li, et al. (2015a). "The functional interactome of PYHIN immune regulators reveals IFIX is a sensor of viral DNA." Mol Syst Biol 11(1): 787.

Diner, B. A., K. K. Lum, et al. (2015b). "Interactions of the antiviral factor IFI16 mediate immune signaling and herpes simplex virus-1 immunosuppression." Mol Cell Proteomics.

Ding, H. F., Y. L. Lin, et al. (2000). "Essential role for caspase-8 in transcription-independent apoptosis triggered by p53." J Biol Chem 275(49): 38905-11.

Ding, Y., J. F. Lee, et al. (2006). "Interferon-inducible protein IFIXalpha1 functions as a negative regulator of HDM2." Mol Cell Biol 26(5): 1979-96.

Ding, Y., L. Wang, et al. (2004). "Antitumor activity of IFIX, a novel interferon-inducible HIN-200 gene, in breast cancer." Oncogene 23(26): 4556-66.

Doil, C., N. Mailand, et al. (2009). "RNF168 binds and amplifies ubiquitin conjugates on damaged chromosomes to allow accumulation of repair proteins." Cell 136(3): 435-46.

Duan, X., L. Ponomareva, et al. (2011). "IFI16 induction by glucose restriction in human fibroblasts contributes to autophagy through activation of the ATM/AMPK/p53 pathway." PLoS One 6(5): e19532.

Dubin, G., N. O. Fishman, et al. (1992). "The role of herpes simplex virus glycoproteins in immune evasion." Curr Top Microbiol Immunol 179: 111-20.

Dubin, G., I. Frank, et al. (1990). "Herpes simplex virus type 1 encodes two Fc receptors which have different binding characteristics for monomeric immunoglobulin G (IgG) and IgG complexes." J Virol 64(6): 2725-31.

Dutta, D., S. Dutta, et al. (2015). "BRCA1 Regulates IFI16 Mediated Nuclear Innate Sensing of Herpes Viral DNA and Subsequent Induction of the Innate Inflammasome and Interferon-beta Responses." PLoS Pathog 11(6): e1005030.

Egistelli, L., S. Chichiarelli, et al. (2009). "IFI16 and NM23 bind to a common DNA fragment both in

the P53 and the cMYC gene promoters." J Cell Biochem 106(4): 666-72.
Emeny, J. M. and M. J. Morgan (1979). "Regulation of the interferon system: evidence that Vero cells have a genetic defect in interferon production." J Gen Virol 43(1): 247-52.
Everett, R. D. (1989). "Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1." J Gen Virol 70 (Pt 5): 1185-202.
Everett, R. D. (2015). "Dynamic Response of IFI16 and Promyelocytic Leukemia Nuclear Body Components to Herpes Simplex Virus 1 Infection." J Virol 90(1): 167-79.
Everett, R. D., C. Boutell, et al. (2004). "Phenotype of a herpes simplex virus type 1 mutant that fails to express immediate-early regulatory protein ICP0." J Virol 78(4): 1763-74.
Everett, R. D., P. Freemont, et al. (1998). "The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms." J Virol 72(8): 6581-91.
Everett, R. D. and G. G. Maul (1994). "HSV-1 IE protein Vmw110 causes redistribution of PML." EMBO J 13(21): 5062-9.
Everett, R. D. and J. Murray (2005). "ND10 components relocate to sites associated with herpes simplex virus type 1 nucleoprotein complexes during virus infection." J Virol 79(8): 5078-89.
Feng, Z., L. Kachnic, et al. (2004). "DNA damage induces p53-dependent BRCA1 nuclear export." J Biol Chem 279(27): 28574-84.
Ferguson, B. J., D. S. Mansur, et al. (2012). "DNA-PK is a DNA sensor for IRF-3-dependent innate immunity." Elife 1: e00047.
Fernandes-Alnemri, T., J. W. Yu, et al. (2009). "AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA." Nature 458(7237): 509-13.
Fields, B. N., Knipe, D.M., Chanock, R.M., Hirsch, M.S., Melnick, J.L., Monath, T.P., Roizman, B. (1990). Virology. New York, Raven Press.
Forrester, A., H. Farrell, et al. (1992). "Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted." J Virol 66(1): 341-8.
Fraser, J. A., J. R. Reeves, et al. (2003). "A role for BRCA1 in sporadic breast cancer." Br J Cancer 88(8): 1263-70.
Fridman, A. L. and M. A. Tainsky (2008). "Critical pathways in cellular senescence and immortalization revealed by gene expression profiling." Oncogene 27(46): 5975-87.
Fujiuchi, N., J. A. Aglipay, et al. (2004). "Requirement of IFI16 for the maximal activation of p53 induced by ionizing radiation." J Biol Chem 279(19): 20339-44.
Galanty, Y., R. Belotserkovskaya, et al. (2009). "Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks." Nature 462(7275): 935-9.
Garber, D. A., S. M. Beverley, et al. (1993). "Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis." Virology 197(1): 459-62.
Gariano, G. R., V. Dell'Oste, et al. (2012). "The intracellular DNA sensor IFI16 gene acts as restriction factor for human cytomegalovirus replication." PLoS Pathog 8(1): e1002498.

Gariglio, M., B. Azzimonti, et al. (2002). "Immunohistochemical expression analysis of the human interferon-inducible gene IFI16, a member of the HIN200 family, not restricted to hematopoietic cells." J Interferon Cytokine Res 22(7): 815-21.

Giacinti, C. and A. Giordano (2006). "RB and cell cycle progression." Oncogene 25(38): 5220-7.

Goldenberg, A., H. Masui, et al. (1989). "Imaging of human tumor xenografts with an indium-111-labeled anti-epidermal growth factor receptor monoclonal antibody." J Natl Cancer Inst 81(21): 1616-25.

Goodbourn, S., L. Didcock, et al. (2000). "Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures." J Gen Virol 81(Pt 10): 2341-64.

Gudas, J. M., T. Li, et al. (1996). "Cell cycle regulation of BRCA1 messenger RNA in human breast epithelial cells." Cell Growth Differ 7(6): 717-23.

Gudas, J. M., H. Nguyen, et al. (1995). "Hormone-dependent regulation of BRCA1 in human breast cancer cells." Cancer Res 55(20): 4561-5.

Gugliesi, F., M. De Andrea, et al. (2010). "The proapoptotic activity of the Interferon-inducible gene IFI16 provides new insights into its etiopathogenetic role in autoimmunity." J Autoimmun 35(2): 114-23.

Gugliesi, F., M. Mondini, et al. (2005). "Up-regulation of the interferon-inducible IFI16 gene by oxidative stress triggers p53 transcriptional activity in endothelial cells." J Leukoc Biol 77(5): 820-9.

Gutterman, J. U. (1994). "Cytokine therapeutics: lessons from interferon alpha." Proc Natl Acad Sci U S A 91(4): 1198-205.

Hamerman, J. A., K. Ogasawara, et al. (2004). "Cutting edge: Toll-like receptor signaling in macrophages induces ligands for the NKG2D receptor." J Immunol 172(4): 2001-5.

Hancock, M. H., A. R. Cliffe, et al. (2010). "Herpes simplex virus VP16, but not ICP0, is required to reduce histone occupancy and enhance histone acetylation on viral genomes in U2OS osteosarcoma cells." J Virol 84(3): 1366-75.

Hanke, T., F. L. Graham, et al. (1990). "Herpes simplex virus IgG Fc receptors induced using recombinant adenovirus vectors expressing glycoproteins E and I." Virology 177(2): 437-44.

Harkin, D. P., J. M. Bean, et al. (1999). "Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1." Cell 97(5): 575-86.

Haupt, Y., R. Maya, et al. (1997). "Mdm2 promotes the rapid degradation of p53." Nature 387(6630): 296-9.

Haupt, Y., S. Rowan, et al. (1995). "Induction of apoptosis in HeLa cells by trans-activation-deficient p53." Genes Dev 9(17): 2170-83.

Hemmi, H., O. Takeuchi, et al. (2000). "A Toll-like receptor recognizes bacterial DNA." Nature 408(6813): 740-5.

Hill, A., P. Jugovic, et al. (1995). "Herpes simplex virus turns off the TAP to evade host immunity." Nature 375(6530): 411-5.

Hiller, S., A. Kohl, et al. (2003). "NMR structure of the apoptosis- and inflammation-related NALP1

pyrin domain." Structure 11(10): 1199-205.
Hollenberg, M. D. and P. Cuatrecasas (1973). "Epidermal growth factor: receptors in human fibroblasts and modulation of action by cholera toxin." Proc Natl Acad Sci U S A 70(10): 2964-8.
Hollenberg, M. D. and P. Cuatrecasas (1975). "Insulin and epidermal growth factor. Human fibroblast receptors related to deoxyribonucleic acid synthesis and amino acid uptake." J Biol Chem 250(10): 3845-53.
Holt, J. T., M. E. Thompson, et al. (1996). "Growth retardation and tumour inhibition by BRCA1." Nat Genet 12(3): 298-302.
Honess, R. W. and B. Roizman (1974). "Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins." J Virol 14(1): 8-19.
Horan, K. A., K. Hansen, et al. (2013). "Proteasomal degradation of herpes simplex virus capsids in macrophages releases DNA to the cytosol for recognition by DNA sensors." J Immunol 190(5): 2311-9.
Hornung, V., A. Ablasser, et al. (2009). "AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC." Nature 458(7237): 514-8.
Hoving, J. C., G. J. Wilson, et al. (2014). "Signalling C-type lectin receptors, microbial recognition and immunity." Cell Microbiol 16(2): 185-94.
Hsieh, J. C., R. Kuta, et al. (2014). "Identification of two novel functional p53 responsive elements in the herpes simplex virus-1 genome." Virology 460-461: 45-54.
Hsu, L. C. and R. L. White (1998). "BRCA1 is associated with the centrosome during mitosis." Proc Natl Acad Sci U S A 95(22): 12983-8.
Huen, M. S., R. Grant, et al. (2007). "RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly." Cell 131(5): 901-14.
Hummer, B. T., X. L. Li, et al. (2001). "Role for p53 in gene induction by double-stranded RNA." J Virol 75(16): 7774-7.
Hung, M. C. and W. Link (2011). "Protein localization in disease and therapy." J Cell Sci 124(Pt 20): 3381-92.
Imakado, S., T. Hoashi, et al. (1998). "The anti-BRCA1 peptide antibody C-20 recognizes smooth muscle cells." Acta Derm Venereol 78(5): 396-7.
Ishii, K. J., C. Coban, et al. (2006). "A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA." Nat Immunol 7(1): 40-8.
Ishikawa, H. and G. N. Barber (2008). "STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling." Nature 455(7213): 674-8.
Ishikawa, H., Z. Ma, et al. (2009). "STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity." Nature 461(7265): 788-92.
Ivashkiv, L. B. and L. T. Donlin (2014). "Regulation of type I interferon responses." Nat Rev Immunol 14(1): 36-49.
Jackson, S. P. and J. Bartek (2009). "The DNA-damage response in human biology and disease."

Nature 461(7267): 1071-8.
Jakobsen, M. R., R. O. Bak, et al. (2013). "IFI16 senses DNA forms of the lentiviral replication cycle and controls HIV-1 replication." Proc Natl Acad Sci U S A 110(48): E4571-80.
Jensen, R. A., M. E. Thompson, et al. (1996). "BRCA1 is secreted and exhibits properties of a granin." Nat Genet 12(3): 303-8.
Jette, N. and S. P. Lees-Miller (2015). "The DNA-dependent protein kinase: A multifunctional protein kinase with roles in DNA double strand break repair and mitosis." Prog Biophys Mol Biol 117(2-3): 194-205.
Jiang, J., E. S. Yang, et al. (2011). "p53-dependent BRCA1 nuclear export controls cellular susceptibility to DNA damage." Cancer Res 71(16): 5546-57.
Jin, T., A. Perry, et al. (2012). "Structures of the HIN domain:DNA complexes reveal ligand binding and activation mechanisms of the AIM2 inflammasome and IFI16 receptor." Immunity 36(4): 561-71.
Joerger, A. C. and A. R. Fersht (2007). "Structure-function-rescue: the diverse nature of common p53 cancer mutants." Oncogene 26(15): 2226-42.
Johnson, D. C. and V. Feenstra (1987). "Identification of a novel herpes simplex virus type 1-induced glycoprotein which complexes with gE and binds immunoglobulin." J Virol 61(7): 2208-16.
Johnson, D. C., M. C. Frame, et al. (1988). "Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI." J Virol 62(4): 1347-54.
Johnson, K. E., V. Bottero, et al. (2014). "IFI16 restricts HSV-1 replication by accumulating on the hsv-1 genome, repressing HSV-1 gene expression, and directly or indirectly modulating histone modifications." PLoS Pathog 10(11): e1004503.
Johnson, K. E., L. Chikoti, et al. (2013). "Herpes simplex virus 1 infection induces activation and subsequent inhibition of the IFI16 and NLRP3 inflammasomes." J Virol 87(9): 5005-18.
Johnstone, R. W., J. A. Kerry, et al. (1998a). "The human interferon-inducible protein, IFI 16, is a repressor of transcription." J Biol Chem 273(27): 17172-7.
Johnstone, R. W., M. H. Kershaw, et al. (1998b). "Isotypic variants of the interferon-inducible transcriptional repressor IFI 16 arise through differential mRNA splicing." Biochemistry 37(34): 11924-31.
Johnstone, R. W., W. Wei, et al. (2000). "Functional interaction between p53 and the interferon-inducible nucleoprotein IFI 16." Oncogene 19(52): 6033-42.
Kach, J., S. D. Conzen, et al. (2015). "Targeting the glucocorticoid receptor in breast and prostate cancers." Sci Transl Med 7(305): 305ps19.
Kalamvoki, M. and B. Roizman (2014). "HSV-1 degrades, stabilizes, requires, or is stung by STING depending on ICP0, the US3 protein kinase, and cell derivation." Proc Natl Acad Sci U S A 111(5): E611-7.
Kaplan, O., J. W. Jaroszewski, et al. (1990). "Toxicity and effects of epidermal growth factor on glucose metabolism of MDA-468 human breast cancer cells." J Biol Chem 265(23): 13641-9.
Kawai, T., S. Sato, et al. (2004). "Interferon-alpha induction through Toll-like receptors involves a

direct interaction of IRF7 with MyD88 and TRAF6." Nat Immunol 5(10): 1061-8.
Kawai, T., K. Takahashi, et al. (2005). "IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction." Nat Immunol 6(10): 981-8.
Kent, J. R., P. Y. Zeng, et al. (2004). "During lytic infection herpes simplex virus type 1 is associated with histones bearing modifications that correlate with active transcription." J Virol 78(18): 10178-86.
Kerur, N., M. V. Veettil, et al. (2011). "IFI16 Acts as a Nuclear Pathogen Sensor to Induce the Inflammasome in Response to Kaposi Sarcoma-Associated Herpesvirus Infection." Cell Host Microbe 9(5): 363-75.
Khare, S., R. A. Ratsimandresey, et al. (2014). "The PYRIN domain-only protein POP3 inhibits ALR inflammasomes and regulates responses to infection with DNA viruses." Nat Immunol 15(4): 343-53.
Kim, E. J., J. I. Park, et al. (2005). "IFI16 is an essential mediator of growth inhibition, but not differentiation, induced by the leukemia inhibitory factor/JAK/STAT pathway in medullary thyroid carcinoma cells." J Biol Chem 280(6): 4913-20.
Kim, T., S. Pazhoor, et al. (2010). "Aspartate-glutamate-alanine-histidine box motif (DEAH)/RNA helicase A helicases sense microbial DNA in human plasmacytoid dendritic cells." Proc Natl Acad Sci U S A 107(34): 15181-6.
Kingsmore, S. F., J. Snoddy, et al. (1989). "Physical mapping of a family of interferon-activated genes, serum amyloid P-component, and alpha-spectrin on mouse chromosome 1." Immunogenetics 30(3): 169-74.
Kloss, M., P. Decker, et al. (2008). "Interaction of monocytes with NK cells upon Toll-like receptor-induced expression of the NKG2D ligand MICA." J Immunol 181(10): 6711-9.
Klupp, B. G., H. Granzow, et al. (2001). "Pseudorabies virus UL37 gene product is involved in secondary envelopment." J Virol 75(19): 8927-36.
Kolas, N. K., J. R. Chapman, et al. (2007). "Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase." Science 318(5856): 1637-40.
Kondo, T., J. Kobayashi, et al. (2013). "DNA damage sensor MRE11 recognizes cytosolic double-stranded DNA and induces type I interferon by regulating STING trafficking." Proc Natl Acad Sci U S A 110(8): 2969-74.
Kondo, Y., K. Nagai, et al. (2012). "Overexpression of the DNA sensor proteins, absent in melanoma 2 and interferon-inducible 16, contributes to tumorigenesis of oral squamous cell carcinoma with p53 inactivation." Cancer Sci 103(4): 782-90.
Koul, D., N. U. Obeyesekere, et al. (1998). "p202 self-associates through a sequence conserved among the members of the 200-family proteins." FEBS Lett 438(1-2): 21-4.
Kruse, J. P. and W. Gu (2009). "Modes of p53 regulation." Cell 137(4): 609-22.
Kute, T. E. and Y. Quadri (1991). "Measurement of proliferation nuclear and membrane markers in tumor cells by flow cytometry." J Histochem Cytochem 39(8): 1125-30.
Kwak, J. C., P. P. Ongusaha, et al. (2003). "IFI16 as a negative regulator in the regulation of p53 and p21(Waf1)." J Biol Chem 278(42): 40899-904.

La Boissiere, S., T. Hughes, et al. (1999). "HCF-dependent nuclear import of VP16." EMBO J 18(2): 480-9.

Lakin, N. D. and S. P. Jackson (1999). "Regulation of p53 in response to DNA damage." Oncogene 18(53): 7644-55.

Lanfranca, M. P., H. H. Mostafa, et al. (2014). "HSV-1 ICP0: An E3 Ubiquitin Ligase That Counteracts Host Intrinsic and Innate Immunity." Cells 3(2): 438-54.

Laptenko, O. and C. Prives (2006). "Transcriptional regulation by p53: one protein, many possibilities." Cell Death Differ 13(6): 951-61.

Latz, E., A. Schoenemeyer, et al. (2004). "TLR9 signals after translocating from the ER to CpG DNA in the lysosome." Nat Immunol 5(2): 190-8.

Lavin, M. F. and N. Gueven (2006). "The complexity of p53 stabilization and activation." Cell Death Differ 13(6): 941-50.

Lees-Miller, S. P., M. C. Long, et al. (1996). "Attenuation of DNA-dependent protein kinase activity and its catalytic subunit by the herpes simplex virus type 1 transactivator ICP0." J Virol 70(11): 7471-7.

Levine, A. J. and M. Oren (2009). "The first 30 years of p53: growing ever more complex." Nat Rev Cancer 9(10): 749-58.

Li, H., R. Baskaran, et al. (2008). "Chk2 is required for HSV-1 ICP0-mediated G2/M arrest and enhancement of virus growth." Virology 375(1): 13-23.

Li, T., J. Chen, et al. (2013). "Human cytomegalovirus tegument protein pUL83 inhibits IFI16-mediated DNA sensing for immune evasion." Cell Host Microbe 14(5): 591-9.

Li, T., B. A. Diner, et al. (2012). "Acetylation modulates cellular distribution and DNA sensing ability of interferon-inducible protein IFI16." Proc Natl Acad Sci U S A 109(26): 10558-63.

Li, T., B. A. Diner, et al. (2012a). "Acetylation modulates cellular distribution and DNA sensing ability of interferon-inducible protein IFI16." Proc Natl Acad Sci U S A 109(26): 10558-63.

Li, Y., R. Chen, et al. (2012b). "LSm14A is a processing body-associated sensor of viral nucleic acids that initiates cellular antiviral response in the early phase of viral infection." Proc Natl Acad Sci U S A 109(29): 11770-5.

Liao, J. C., R. Lam, et al. (2011). "Interferon-inducible protein 16: insight into the interaction with tumor suppressor p53." Structure 19(3): 418-29.

Liepinsh, E., R. Barbals, et al. (2003). "The death-domain fold of the ASC PYRIN domain, presenting a basis for PYRIN/PYRIN recognition." J Mol Biol 332(5): 1155-63.

Lilley, C. E., C. T. Carson, et al. (2005). "DNA repair proteins affect the lifecycle of herpes simplex virus 1." Proc Natl Acad Sci U S A 102(16): 5844-9.

Lilley, C. E., M. S. Chaurushiya, et al. (2011). "The intrinsic antiviral defense to incoming HSV-1 genomes includes specific DNA repair proteins and is counteracted by the viral protein ICP0." PLoS Pathog 7(6): e1002084.

Lilley, C. E., M. S. Chaurushiya, et al. (2010). "A viral E3 ligase targets RNF8 and RNF168 to control

histone ubiquitination and DNA damage responses." EMBO J 29(5): 943-55.
Lilley, C. E., R. A. Schwartz, et al. (2007). "Using or abusing: viruses and the cellular DNA damage response." Trends Microbiol 15(3): 119-26.
Liu, D. P., H. Song, et al. (2010). "A common gain of function of p53 cancer mutants in inducing genetic instability." Oncogene 29(7): 949-56.
Ljungman, M. (2000). "Dial 9-1-1 for p53: mechanisms of p53 activation by cellular stress." Neoplasia 2(3): 208-25.
Ljungman, M. and D. P. Lane (2004). "Transcription - guarding the genome by sensing DNA damage." Nat Rev Cancer 4(9): 727-37.
Loo, Y. M. and M. Gale, Jr. (2011). "Immune signaling by RIG-I-like receptors." Immunity 34(5): 680-92.
Lopez-Knowles, E., S. Hernandez, et al. (2006). "PIK3CA mutations are an early genetic alteration associated with FGFR3 mutations in superficial papillary bladder tumors." Cancer Res 66(15): 7401-4.
Loughery, J., M. Cox, et al. (2014). "Critical role for p53-serine 15 phosphorylation in stimulating transactivation at p53-responsive promoters." Nucleic Acids Res 42(12): 7666-80.
Lu, M., S. D. Conzen, et al. (1996). "Characterization of functional messenger RNA splice variants of BRCA1 expressed in nonmalignant and tumor-derived breast cells." Cancer Res 56(20): 4578-81.
Ludlow, L. E., R. W. Johnstone, et al. (2005). "The HIN-200 family: more than interferon-inducible genes?" Exp Cell Res 308(1): 1-17.
Lukashchuk, V. and R. D. Everett (2010). "Regulation of ICP0-null mutant herpes simplex virus type 1 infection by ND10 components ATRX and hDaxx." J Virol 84(8): 4026-40.
Luu, P. and O. Flores (1997). "Binding of SP1 to the immediate-early protein-responsive element of the human cytomegalovirus DNA polymerase promoter." J Virol 71(9): 6683-91.
Mailand, N., S. Bekker-Jensen, et al. (2007). "RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins." Cell 131(5): 887-900.
Mangia, A., A. Chiriatti, et al. (2009). "BRCA1 expression and molecular alterations in familial breast cancer." Histol Histopathol 24(1): 69-76.
Maul, G. G., H. H. Guldner, et al. (1993). "Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0)." J Gen Virol 74 (Pt 12): 2679-90.
Maul, G. G., D. E. Jensen, et al. (1998). "Nuclear redistribution of BRCA1 during viral infection." Cell Growth Differ 9(9): 743-55.
McFarlane, M., J. I. Daksis, et al. (1992). "Hexamethylene bisacetamide stimulates herpes simplex virus immediate early gene expression in the absence of trans-induction by Vmw65." J Gen Virol 73 (Pt 2): 285-92.
McSharry, B. P., C. J. Jones, et al. (2001). "Human telomerase reverse transcriptase-immortalized MRC-5 and HCA2 human fibroblasts are fully permissive for human cytomegalovirus." J Gen Virol 82(Pt 4): 855-63.
Medzhitov, R. (2007). "Recognition of microorganisms and activation of the immune response."

Nature 449(7164): 819-26.
Mettenleiter, T. C., B. G. Klupp, et al. (2009). “Herpesvirus assembly: an update.” Virus Res 143(2): 222-34.
Mettenleiter, T. C., F. Muller, et al. (2013). “The way out: what we know and do not know about herpesvirus nuclear egress.” Cell Microbiol 15(2): 170-8.
Meyer, G. A. and K. D. Radsak (2000). “Identification of a novel signal sequence that targets transmembrane proteins to the nuclear envelope inner membrane.” J Biol Chem 275(6): 3857-66.
Meylan, E., J. Curran, et al. (2005). “Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus.” Nature 437(7062): 1167-72.
Milner, R., H. Wombwell, et al. (2013). “Validation of the BRCA1 antibody MS110 and the utility of BRCA1 as a patient selection biomarker in immunohistochemical analysis of breast and ovarian tumours.” Virchows Arch 462(3): 269-79.
Min, W., S. Ghosh, et al. (1996). “The interferon-inducible p202 protein as a modulator of transcription: inhibition of NF-kappa B, c-Fos, and c-Jun activities.” Mol Cell Biol 16(1): 359-68.
Mohni, K. N., C. M. Livingston, et al. (2010). “ATR and ATRIP are recruited to herpes simplex virus type 1 replication compartments even though ATR signaling is disabled.” J Virol 84(23): 12152-64.
Mondini, M., M. Vidali, et al. (2006). “A novel autoantigen to differentiate limited cutaneous systemic sclerosis from diffuse cutaneous systemic sclerosis: the interferon-inducible gene IFI16.” Arthritis Rheum 54(12): 3939-44.
Moore, M., N. Horikoshi, et al. (1996). “Oncogenic potential of the adenovirus E4orf6 protein.” Proc Natl Acad Sci U S A 93(21): 11295-301.
Mori, T., Y. Anazawa, et al. (2002). “Identification of the interferon regulatory factor 5 gene (IRF-5) as a direct target for p53.” Oncogene 21(18): 2914-8.
Moynahan, M. E., J. W. Chiu, et al. (1999). “Brca1 controls homology-directed DNA repair.” Mol Cell 4(4): 511-8.
Munoz-Fontela, C., S. Macip, et al. (2008). “Transcriptional role of p53 in interferon-mediated antiviral immunity.” J Exp Med 205(8): 1929-38.
Muruve, D. A., V. Petrilli, et al. (2008). “The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response.” Nature 452(7183): 103-7.
Nahmias, A. J. and W. R. Dowdle (1968). “Antigenic and biologic differences in herpesvirus hominis.” Prog Med Virol 10: 110-59.
Nie, Y. and Y. Y. Wang (2013). “Innate immune responses to DNA viruses.” Protein Cell 4(1): 1-7.
O’Brate, A. and P. Giannakakou (2003). “The importance of p53 location: nuclear or cytoplasmic zip code?” Drug Resist Updat 6(6): 313-22.
O’Neill, L. A., D. Golenbock, et al. (2013). “The history of Toll-like receptors - redefining innate immunity.” Nat Rev Immunol 13(6): 453-60.
Oh, J. and N. W. Fraser (2008). “Temporal association of the herpes simplex virus genome with histone proteins during a lytic infection.” J Virol 82(7): 3530-7.

Olivier, M., M. Hollstein, et al. (2010). “TP53 mutations in human cancers: origins, consequences, and clinical use.” Cold Spring Harb Perspect Biol 2(1): a001008.
Opdenakker, G., J. Snoddy, et al. (1989). “Interferons as gene activators: a cluster of six interferon-activatable genes is linked to the erythroid alpha-spectrin locus on murine chromosome 1.” Virology 171(2): 568-78.
Orzalli, M. H., N. M. Broekema, et al. (2015). “cGAS-mediated stabilization of IFI16 promotes innate signaling during herpes simplex virus infection.” Proc Natl Acad Sci U S A 112(14): E1773-81.
Orzalli, M. H., S. E. Conwell, et al. (2013). “Nuclear interferon-inducible protein 16 promotes silencing of herpesviral and transfected DNA.” Proc Natl Acad Sci U S A 110(47): E4492-501.
Orzalli, M. H., N. A. DeLuca, et al. (2012). “Nuclear IFI16 induction of IRF-3 signaling during herpesviral infection and degradation of IFI16 by the viral ICP0 protein.” Proc Natl Acad Sci U S A 109(44): E3008-17.
Orzalli, M. H. and D. M. Knipe (2014). “Cellular sensing of viral DNA and viral evasion mechanisms.” Annu Rev Microbiol 68: 477-92.
Paludan, S. R. and A. G. Bowie (2013). “Immune sensing of DNA.” Immunity 38(5): 870-80.
Pampin, M., Y. Simonin, et al. (2006). “Cross talk between PML and p53 during poliovirus infection: implications for antiviral defense.” J Virol 80(17): 8582-92.
Parkinson, J., S. P. Lees-Miller, et al. (1999). “Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase.” J Virol 73(1): 650-7.
Parvatiyar, K., Z. Zhang, et al. (2012). “The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response.” Nat Immunol 13(12): 1155-61.
Pawlowski, K., F. Pio, et al. (2001). “PAAD - a new protein domain associated with apoptosis, cancer and autoimmune diseases.” Trends Biochem Sci 26(2): 85-7.
Perez-Valles, A., M. Martorell-Cebollada, et al. (2001). “The usefulness of antibodies to the BRCA1 protein in detecting the mutated BRCA1 gene. An immunohistochemical study.” J Clin Pathol 54(6): 476-80.
Placek, B. J. and S. L. Berger (2010). “Chromatin dynamics during herpes simplex virus-1 lytic infection.” Biochim Biophys Acta 1799(3-4): 223-7.
Preston, C. M. (1979). “Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK.” J Virol 29(1): 275-84.
Preston, C. M. and M. McFarlane (1998). “Cytodifferentiating agents affect the replication of herpes simplex virus type 1 in the absence of functional VP16.” Virology 249(2): 418-26.
Preston, C. M., A. Rinaldi, et al. (1998). “Herpes simplex virus type 1 immediate early gene expression is stimulated by inhibition of protein synthesis.” J Gen Virol 79 (Pt 1): 117-24.
Querido, E., P. Blanchette, et al. (2001). “Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex.” Genes Dev 15(23):

3104-17.
Querido, E., R. C. Marcellus, et al. (1997). “Regulation of p53 levels by the E1B 55-kilodalton protein and E4orf6 in adenovirus-infected cells.” J Virol 71(5): 3788-98.
Raffaella, R., D. Gioia, et al. (2004). “The interferon-inducible IFI16 gene inhibits tube morphogenesis and proliferation of primary, but not HPV16 E6/E7-immortalized human endothelial cells.” Exp Cell Res 293(2): 331-45.
Rathinam, V. A., S. K. Vanaja, et al. (2012). “Regulation of inflammasome signaling.” Nat Immunol 13(4): 333-42.
Reske, A., G. Pollara, et al. (2007). “Understanding HSV-1 entry glycoproteins.” Rev Med Virol 17(3): 205-15.
Rhim, J. S., K. Schell, et al. (1969). “Biological characteristics and viral susceptibility of an African green monkey kidney cell line (Vero).” Proc Soc Exp Biol Med 132(2): 670-8.
Rivas, C., S. A. Aaronson, et al. (2010). “Dual Role of p53 in Innate Antiviral Immunity.” Viruses 2(1): 298-313.
Roizman, B., L. E. Carmichael, et al. (1981). “Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses.” Intervirology 16(4): 201-17.
Rosen, E. M., S. Fan, et al. (2006). “BRCA1 regulation of transcription.” Cancer Lett 236(2): 175-85.
Sadler, A. J. and B. R. Williams (2008). “Interferon-inducible antiviral effectors.” Nat Rev Immunol 8(7): 559-68.
Samaniego, L. A., L. Neiderhiser, et al. (1998). “Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins.” J Virol 72(4): 3307-20.
Samanta, H., D. A. Engel, et al. (1986). “Interferons as gene activators. Cloning of the 5’ terminus and the control segment of an interferon activated gene.” J Biol Chem 261(25): 11849-58.
Scheffner, M., B. A. Werness, et al. (1990). “The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53.” Cell 63(6): 1129-36.
Scully, R., J. Chen, et al. (1997b). “Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage.” Cell 90(3): 425-35.
Scully, R., J. Chen, et al. (1997a). “Association of BRCA1 with Rad51 in mitotic and meiotic cells.” Cell 88(2): 265-75.
Scully, R., S. Ganesan, et al. (1996). “Location of BRCA1 in human breast and ovarian cancer cells.” Science 272(5258): 123-6.
Seelig, H. P., H. Ehrfeld, et al. (1994). “Interferon-gamma-inducible protein p16. A new target of antinuclear antibodies in patients with systemic lupus erythematosus.” Arthritis Rheum 37(11): 1672-83.
Serrano, M., A. W. Lin, et al. (1997). “Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.” Cell 88(5): 593-602.
Seth, R. B., L. Sun, et al. (2005). “Identification and characterization of MAVS, a mitochondrial

antiviral signaling protein that activates NF-kappaB and IRF 3." Cell 122(5): 669-82.
Shao, N., Y. L. Chai, et al. (1996). "Induction of apoptosis by the tumor suppressor protein BRCA1." Oncogene 13(1): 1-7.
Sharma, A. K., K. Horgan, et al. (1994). "A dual immunocytochemical assay for oestrogen and epidermal growth factor receptors in tumour cell lines." Histochem J 26(4): 306-10.
Shirata, N., A. Kudoh, et al. (2005). "Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection." J Biol Chem 280(34): 30336-41.
Sigal, A. and V. Rotter (2000). "Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome." Cancer Res 60(24): 6788-93.
Singh, V. V., N. Kerur, et al. (2013). "Kaposi's sarcoma-associated herpesvirus latency in endothelial and B cells activates gamma interferon-inducible protein 16-mediated inflammasomes." J Virol 87(8): 4417-31.
Skaliter, R. and I. R. Lehman (1994). "Rolling circle DNA replication in vitro by a complex of herpes simplex virus type 1-encoded enzymes." Proc Natl Acad Sci U S A 91(22): 10665-9.
Smiley, J. R., M. M. Elgadi, et al. (2001). "Herpes simplex virus vhs protein." Methods Enzymol 342: 440-51.
Smith, G. C. and S. P. Jackson (1999). "The DNA-dependent protein kinase." Genes Dev 13(8): 916-34.
Sodeik, B., M. W. Ebersold, et al. (1997). "Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus." J Cell Biol 136(5): 1007-21.
Song, L. L., F. Alimirah, et al. (2008). "Expression of an IFN-inducible cellular senescence gene, IFI16, is up-regulated by p53." Mol Cancer Res 6(11): 1732-41.
Stamps, A. C., S. C. Davies, et al. (1994). "Analysis of proviral integration in human mammary epithelial cell lines immortalized by retroviral infection with a temperature-sensitive SV40 T-antigen construct." Int J Cancer 57(6): 865-74.
Stark, J. M., A. J. Pierce, et al. (2004). "Genetic steps of mammalian homologous repair with distinct mutagenic consequences." Mol Cell Biol 24(21): 9305-16.
Staub, E., E. Dahl, et al. (2001). "The DAPIN family: a novel domain links apoptotic and interferon response proteins." Trends Biochem Sci 26(2): 83-5.
Steegenga, W. T., N. Riteco, et al. (1998). "The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells." Oncogene 16(3): 349-57.
Stetson, D. B. and R. Medzhitov (2006). "Recognition of cytosolic DNA activates an IRF3-dependent innate immune response." Immunity 24(1): 93-103.
Stow, N. D. and E. C. Stow (1986). "Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110." J Gen Virol 67 (Pt 12): 2571-85.
Strang, B. L. and N. D. Stow (2005). "Circularization of the herpes simplex virus type 1 genome upon

lytic infection." J Virol 79(19): 12487-94.
Sun, L., J. Wu, et al. (2013). "Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway." Science 339(6121): 786-91.
Taddeo, B., T. R. Luo, et al. (2003). "Activation of NF-kappaB in cells productively infected with HSV-1 depends on activated protein kinase R and plays no apparent role in blocking apoptosis." Proc Natl Acad Sci U S A 100(21): 12408-13.
Taddeo, B., W. Zhang, et al. (2004). "Cells lacking NF-kappaB or in which NF-kappaB is not activated vary with respect to ability to sustain herpes simplex virus 1 replication and are not susceptible to apoptosis induced by a replication-incompetent mutant virus." J Virol 78(21): 11615-21.
Takaoka, A., S. Hayakawa, et al. (2003). "Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence." Nature 424(6948): 516-23.
Takaoka, A., Z. Wang, et al. (2007). "DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response." Nature 448(7152): 501-5.
Tanaka, Y. and Z. J. Chen (2012). "STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway." Sci Signal 5(214): ra20.
Taura, M., A. Eguma, et al. (2008). "p53 regulates Toll-like receptor 3 expression and function in human epithelial cell lines." Mol Cell Biol 28(21): 6557-67.
Tavalai, N. and T. Stamminger (2008). "New insights into the role of the subnuclear structure ND10 for viral infection." Biochim Biophys Acta 1783(11): 2207-21.
Taylor, T. J. and D. M. Knipe (2004). "Proteomics of herpes simplex virus replication compartments: association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8." J Virol 78(11): 5856-66.
Thakur, S., H. B. Zhang, et al. (1997). "Localization of BRCA1 and a splice variant identifies the nuclear localization signal." Mol Cell Biol 17(1): 444-52.
Thomas, J. E., M. Smith, et al. (1996). "Subcellular localization and analysis of apparent 180-kDa and 220-kDa proteins of the breast cancer susceptibility gene, BRCA1." J Biol Chem 271(45): 28630-5.
Thompson, M. E. (2010). "BRCA1 16 years later: nuclear import and export processes." FEBS J 277(15): 3072-8.
Tokunaga, T., O. Yano, et al. (1992). "Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of Mycobacterium bovis BCG induce interferons and activate natural killer cells." Microbiol Immunol 36(1): 55-66.
Tomlinson, G. E., T. T. Chen, et al. (1998). "Characterization of a breast cancer cell line derived from a germ-line BRCA1 mutation carrier." Cancer Res 58(15): 3237-42.
Townsend, P. A., T. M. Scarabelli, et al. (2004). "STAT-1 interacts with p53 to enhance DNA damage-induced apoptosis." J Biol Chem 279(7): 5811-20.
Trapani, J. A., K. A. Browne, et al. (1992). "A novel gene constitutively expressed in human lymphoid cells is inducible with interferon-gamma in myeloid cells." Immunogenetics 36(6): 369-76.
Tulchin, N., M. Chambon, et al. (2010). "BRCA1 protein and nucleolin colocalize in breast carcinoma

tissue and cancer cell lines." Am J Pathol 176(3): 1203-14.
Turnell, A. S. and R. J. Grand (2012). "DNA viruses and the cellular DNA-damage response." J Gen Virol 93(Pt 10): 2076-97.
Uchida, K., Y. Akita, et al. (2005). "Identification of specific autoantigens in Sjogren's syndrome by SEREX." Immunology 116(1): 53-63.
Unterholzner, L. and A. G. Bowie (2011). "Innate DNA sensing moves to the nucleus." Cell Host Microbe 9(5): 351-3.
Unterholzner, L., S. E. Keating, et al. (2010). "IFI16 is an innate immune sensor for intracellular DNA." Nat Immunol 11(11): 997-1004.
Veeranki, S. and D. Choubey (2011). "Interferon-inducible p200-family protein IFI16, an innate immune sensor for cytosolic and nuclear double-stranded DNA: regulation of subcellular localization." Mol Immunol 49(4): 567-71.
Wang, B. and S. J. Elledge (2007). "Ubc13/Rnf8 ubiquitin ligases control foci formation of the Rap80/Abraxas/Brca1/Brcc36 complex in response to DNA damage." Proc Natl Acad Sci U S A 104(52): 20759-63.
Wang, H. C., W. C. Chou, et al. (2006). "Ataxia telangiectasia mutated and checkpoint kinase 2 regulate BRCA1 to promote the fidelity of DNA end-joining." Cancer Res 66(3): 1391-400.
Wang, Y., D. Cortez, et al. (2000). "BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures." Genes Dev 14(8): 927-39.
Watson, R. J. and J. B. Clements (1978). "Characterization of transcription-deficient temperature-sensitive mutants of herpes simplex virus type 1." Virology 91(2): 364-79.
Wei, W., C. J. Clarke, et al. (2003). "Expression of IFI 16 in epithelial cells and lymphoid tissues." Histochem Cell Biol 119(1): 45-54.
Weinheimer, S. P., B. A. Boyd, et al. (1992). "Deletion of the VP16 open reading frame of herpes simplex virus type 1." J Virol 66(1): 258-69.
Weir, J. P. (2001). "Regulation of herpes simplex virus gene expression." Gene 271(2): 117-30.
Weitzman, M. D., C. E. Lilley, et al. (2010). "Genomes in conflict: maintaining genome integrity during virus infection." Annu Rev Microbiol 64: 61-81.
Weller, S. K. and D. M. Coen (2012). "Herpes simplex viruses: mechanisms of DNA replication." Cold Spring Harb Perspect Biol 4(9): a013011.
Wilcock, D. and D. P. Lane (1991). "Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells." Nature 349(6308): 429-31.
Wildy, P. (1973). Herpes: history and classification. The herpesviruses. A. Kaplan. New York, Academic Press. 1.
Wilson, C. A., M. N. Payton, et al. (1997). "Differential subcellular localization, expression and biological toxicity of BRCA1 and the splice variant BRCA1-delta11b." Oncogene 14(1): 1-16.
Wilson, C. A., M. N. Payton, et al. (1996). "BRCA1 protein products: antibody specificity." Nat Genet 13(3): 264-5.

Wilson, C. A., L. Ramos, et al. (1999). "Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinomas." Nat Genet 21(2): 236-40.
Wu, J., L. Y. Lu, et al. (2010). "The role of BRCA1 in DNA damage response." Protein Cell 1(2): 117-23.
Wu, J., L. Sun, et al. (2013). "Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA." Science 339(6121): 826-30.
Wu, W., A. Koike, et al. (2008). "The ubiquitin E3 ligase activity of BRCA1 and its biological functions." Cell Div 3: 1.
Wysocka, J. and W. Herr (2003). "The herpes simplex virus VP16-induced complex: the makings of a regulatory switch." Trends Biochem Sci 28(6): 294-304.
Xin, H., J. Curry, et al. (2003). "Role of IFI 16, a member of the interferon-inducible p200-protein family, in prostate epithelial cellular senescence." Oncogene 22(31): 4831-40.
Xin, H., O. M. Pereira-Smith, et al. (2004). "Role of IFI 16 in cellular senescence of human fibroblasts." Oncogene 23(37): 6209-17.
Xu, B., S. Kim, et al. (2001). "Involvement of Brca1 in S-phase and G(2)-phase checkpoints after ionizing irradiation." Mol Cell Biol 21(10): 3445-50.
Xu, C. F., J. A. Chambers, et al. (1997). "Mutations and alternative splicing of the BRCA1 gene in UK breast/ovarian cancer families." Genes Chromosomes Cancer 18(2): 102-10.
Xu, L. G., Y. Y. Wang, et al. (2005). "VISA is an adapter protein required for virus-triggered IFN-beta signaling." Mol Cell 19(6): 727-40.
Yan, H., K. Dalal, et al. (2008). "RPA nucleic acid-binding properties of IFI16-HIN200." Biochim Biophys Acta 1784(7-8): 1087-97.
Yan, N. and Z. J. Chen (2012). "Intrinsic antiviral immunity." Nat Immunol 13(3): 214-22.
Yanai, H., T. Ban, et al. (2009). "HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses." Nature 462(7269): 99-103.
Yang, P., H. An, et al. (2010). "The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway." Nat Immunol 11(6): 487-94.
Yao, F. and P. A. Schaffer (1995). "An activity specified by the osteosarcoma line U2OS can substitute functionally for ICP0, a major regulatory protein of herpes simplex virus type 1." J Virol 69(10): 6249-58.
Yarden, R. I., S. Pardo-Reoyo, et al. (2002). "BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage." Nat Genet 30(3): 285-9.
York, I. A., C. Roop, et al. (1994). "A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes." Cell 77(4): 525-35.
Yoshikawa, K., K. Honda, et al. (1999). "Reduction of BRCA1 protein expression in Japanese sporadic breast carcinomas and its frequent loss in BRCA1-associated cases." Clin Cancer Res 5(6): 1249-61.
Zhang, J., K. Wang, et al. (2013). "Herpes simplex virus 1 E3 ubiquitin ligase ICP0 protein inhibits tumor necrosis factor alpha-induced NF-kappaB activation by interacting with p65/RelA and p50/NF-

kappaB1." J Virol 87(23): 12935-48.
Zhang, X., T. W. Brann, et al. (2011a). "Cutting edge: Ku70 is a novel cytosolic DNA sensor that induces type III rather than type I IFN." J Immunol 186(8): 4541-5.
Zhang, Z., B. Yuan, et al. (2011b). "The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells." Nat Immunol 12(10): 959-65.
Zhong, B., Y. Yang, et al. (2008). "The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation." Immunity 29(4): 538-50.
Zhong, Q., T. G. Boyer, et al. (2002b). "Deficient nonhomologous end-joining activity in cell-free extracts from Brca1-null fibroblasts." Cancer Res 62(14): 3966-70.
Zhong, Q., C. F. Chen, et al. (2002a). "BRCA1 facilitates microhomology-mediated end joining of DNA double strand breaks." J Biol Chem 277(32): 28641-7.
Zhuang, J., J. Zhang, et al. (2006). "Checkpoint kinase 2-mediated phosphorylation of BRCA1 regulates the fidelity of nonhomologous end-joining." Cancer Res 66(3): 1401-8.